Nature-inspired design of motif-specific antibody scaffolds

James T Koerber¹, Nathan D Thomsen¹, Brett T Hannigan², William F Degrado¹ & James A Wells¹,³

Aberrant changes in post-translational modifications (PTMs) such as phosphate groups underlie a majority of human diseases. However, detection and quantification of PTMs for diagnostic or biomarker applications often require PTM-specific monoclonal antibodies (mAbs), which are challenging to generate using traditional antibody-selection methods. Here we outline a general strategy for producing synthetic, PTM-specific mAbs by engineering a motif-specific ‘hot spot’ into an antibody scaffold. Inspired by a natural phosphate-binding motif, we designed and selected mAb scaffolds with hot spots specific for phosphoserine, phosphothreonine or phosphotyrosine. Crystal structures of the phospho-specific mAbs revealed two distinct modes of phosphoepitope recognition. Our data suggest that each hot spot functions independently of the surrounding scaffold, as phage display antibody libraries using these scaffolds yielded >50 phospho- and target-specific mAbs against 70% of target peptides. Our motif-specific scaffold strategy may provide a general solution for rapid, robust development of anti-PTM mAbs for signaling, diagnostic and therapeutic applications.

Posttranslational modification of proteins by phosphorylation, acetylation and ubiquitination is essential in modulating protein function throughout biology. In particular, phosphorylation is one of the most common regulatory mechanisms in eukaryotes: ~20–30% of all eukaryotic proteins can be phosphorylated by the aggregate activity of >500 kinases. Given the ubiquitous role of phosphorylation in signal transduction, it is not surprising that aberrant phosphorylation either directly causes or is a consequence of many human diseases, such as cancer and neurodegenerative disorders.

Recent advances in phosphoproteomic methods have greatly expanded the number of known phosphorylation sites (>170,000) and identified global phosphorylation changes that occur during disease. Ultimately, the validation of phosphorylation sites (>170,000) and identified global phosphorylation changes that occur during disease. Ultimately, the validation of phosphorylation sites, respectively, compared with <0.05% for tyrosine.

Attempts to generate recombinant phospho-specific mAbs using in vitro selection methods, such as phage display, yeast display and ribosome display, have been even less efficient than immunization methods. Engineered endogenous phosphopeptide-binding domains such as Src-homology-2 (SH2) or forhead-associated (FHA) domains may provide an alternative to mAbs, but the general utility of these non-antibody scaffolds remains to be demonstrated.

Recently, the combination of immunization and phage display was used to isolate a high-affinity phospho-specific mAb from chickens. Although this approach was successful and led to the first phospho-specific mAb structure, it relies upon a low-throughput and time-consuming immunization step. We hypothesize that both immunization and in vitro methods for generating phospho-specific mAbs fail to routinely yield high-quality mAbs because most naive mAbs do not possess any detectable affinity for the small, peptide antigens. In light of these difficulties, we envisioned a structure-guided strategy for generating mAbs that employs mAb scaffolds with engineered pockets tailored to a particular motif. This motif-specific anchoring pocket would provide initial antigen-binding affinity and guide the selection of mAbs targeted to epitopes containing the motif (for example, a pSer- or pTyr-containing peptide). These motif residues, known as ‘hot spots’, contribute a substantial fraction of the binding energy to a protein–protein interaction.

Here we engineer mAb scaffolds with designed binding pockets for pSer, pThr or pTyr residues and thereby make these residues hot spots in the antigen–mAb interaction. Guided by a natural phosphate-binding motif and knowledge of mAb structure-function, we first identified a parent mAb scaffold in which to install the designed pocket in the complementarity-determining regions (CDRs). We then mutated the scaffold to specifically bind pSer, pThr or pTyr and solved the X-ray crystal structures of the phospho-specific mAbs, which account for 90% and 10% of all phosphorylation sites, respectively, compared with <0.05% for tyrosine.

¹Department of Pharmaceutical Chemistry, University of California, San Francisco, California, USA. ²Graduate Group in Genomics and Computational Biology (GCB), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. ³Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California, USA. Correspondence should be addressed to J.A.W. (jim.wells@ucsf.edu).

Received 19 February; accepted 17 July; published online 18 August 2013; doi:10.1038/nbt.2672
crystal structures of the phospho-specific mAb:peptide complexes. In the second step, we constructed two large, diverse, single-chain Fv (scFv) mAb phage display libraries based upon these scaffolds and successfully selected 51 phospho-specific mAbs against seven different pSer- or pThr-containing peptides. These results suggest that the phospho-residue-binding pocket functions relatively independently of additional structural and functional changes in other CDRs of the mAb.

RESULTS

Design of phospho-specific mAb scaffolds

To design a mAb scaffold with a phosphate-binding motif, we drew upon structural knowledge of how protein domains recognize anions, such as phosphate. The most common anion-binding motif, called a nest, occurs within many different protein super-families, such as ATPases and kinases, and consists of three consecutive residues where multiple main-chain amides form hydrogen bonds with the anion (Supplementary Fig. 1a)28. Starting with this ubiquitous motif, we sought to find an existing mAb scaffold into which we could build a similar short, localized loop. We focused our search on 60 anti-peptide mAb structures and manually inspected the CDRs for the desired nest conformation. We identified a region of CDR H2 within a mouse Fab (PDB ID 188i)29 that adopts the desired conformation owing to a hallmark ω2 glycine at 54H (Fig. 1a). Notably, this mAb uses the H2 loop to bind an acidic residue by means of six loop residues that anchor the peptide (52H and 52AH), stabilize the conformation (54H), or confer side-chain specificity (53H, 55H and 56H) (Fig. 1a and Supplementary Table 1). A larger search of all mAb-antigen structures identified eight mAbs that use this loop to bind an aspartate or glutamate in the antigen (Supplementary Fig. 1b).

To characterize this class of mAb-antigen interactions, we synthesized the gene encoding a humanized version of the 188i Fab and cloned this construct into both a phage display and protein expression vector (Supplementary Table 2). The humanized scaffold, which was expressed at yields >3 mg/l in bacteria, bound the peptide with similar affinity as reported for the mouse Fab29. To understand the importance of the Asp-loop (residues 52H–56H) interaction in peptide binding, we performed competition phage enzyme-linked immunoabsorbent assays (ELISAs) to analyze Fab binding to a panel of peptides. ELISA data confirmed that the Asp8 residue of the antigen is a hot spot for binding, as mutations to Ala, Ser, Thr or Tyr substantially reduced Fab binding (>100-fold less) to the peptide (Fig. 1b). We reasoned that the carboxylate group of the Asp8 residue might mimic a phosphorylated residue and thus, the mAb may bind peptides with pSer, pThr or possibly pTyr in place of Asp8. ELISA data confirmed the ability of this Fab to bind peptides containing pSer or pThr, albeit with weak affinities (>2,000 nM) (Fig. 1b and Table 1). No mAb binding to the pTyr peptide was observed, probably owing to its large size. Structural analysis of the peptide:Fab complex suggested that steric clashes with several side chains and the main chain of the CDR were likely responsible for the weak affinities.

Therefore, we constructed three antibody phage display libraries to optimize the CDR region for each phosphorylated residue. The six-residue CDR region (52H–56H) was replaced with random residues (H2 library) or seven random residues (H2+1 library) to relieve steric clashes with the mAb backbone. The third library design was similar to the H2 library, but fixed Gly or Ser at 53H and 54H (GS library). These strategies allowed us to assess the importance of the anchor (52H and 52AH) and conformation (54H) residues as well as alter the specificity residues (53H, 55H and 56H). Using standard phage display methods, we then performed four rounds of selection against pSer, pThr and pTyr peptides. Notably, we observed strong enrichment against each of the pSer, pThr and pTyr peptide targets using all three libraries, except for selections with the H2+1 library against pTyr (Fig. 1c and data not shown).

Characterization of phospho-specific mAb scaffolds

For each phosphopeptide antigen, we isolated single phage clones and sequenced the CDR H2 region for clones that bound to the phosphopeptide by single-point ELISA (data not shown). Selections against the pSer and pThr peptides gave similar sequences and thus were combined into one sequence logo. Sequence logos from the

Table 1  Affinity measurements of mAb scaffolds as determined by Biacore

| Fab   | Peptide | $K_a$ (M$^{-1}$ s$^{-1}$) | $k_{on}$ (s$^{-1}$) | $K_d$ (nM) |
|-------|---------|--------------------------|-------------------|-------------|
| Parent Fab | WT Asp | 3.38 × 10$^6$ | 0.0032 | 9.6 |
| pSer | 3.24 × 10$^6$ | 0.0032 | 9.6 |
| pThr | 4.7 × 10$^4$ | 0.041 | 866 |
| Ser/Thr | 1.0 × 10$^5$ | 0.0075 | 71 |
| pSTAb | pSer | 4.8 × 10$^4$ | 0.0082 | 172 |
| pThr | 2.4 × 10$^4$ | 0.0064 | 232 |
| Ser/Thr | 1.9 × 10$^4$ | 0.070 | 360 |
| pYAb | pTyr | 2.84 × 10$^4$ | 0.249 | 8,700 |
| TyR | 2.84 × 10$^4$ | 0.249 | 8,700 |

*No binding seen by competition ELISAs. Peptide sequences for WT, pSer, pThr and pTyr are GEKKGNYVTDDH, GEKKGNYVTpS8H, GEKKGNYVTpTTH and GEKKGNYVTpYTA, respectively. n.d., not determined.
The sequence frequency logos of the mAb to observe the same level of inhibition. ~20-fold less pTyr peptide was required in inhibition was observed at high concentrations of the unphosphorylated Tyr peptide, but whereas strong inhibition was observed for the phosphorylated peptides. (c) For pYAb, weak inhibition was observed for the pSTAb:pSer, pSTAb:pThr and pYAb:pTyr complexes (pSTAb with sequence STPRGST), and a pTyr-specific scaffold (pYAb with sequence VTYGGRK). We were unable to isolate a pThr scaffold that did not cross-react with the pSer peptide. To determine the phospho-selectivity of these scaffolds, we analyzed binding to the phosphorylated and unphosphorylated peptides by ELISA and surface plasmon resonance optical biosensors (Supplementary Fig. 2). Strikingly, we observed high affinity and selectivity for the phosphorylated peptide in all cases (Fig. 2 and Table 1).

Structural analysis of phosphopeptide recognition
To explore the mode of phosphoepitope recognition, we determined the X-ray structure of four Fab:peptide complexes (pSAb:pSer, pSTAb:pSer, pSTAb:pThr and pYAb:pTyr) as well as the unbound pYAb Fab (Supplementary Tables 3 and 4). We observed strong electron density for the bound peptide in all pSer and pThr structures (Supplementary Fig. 3). For the pYAb Fab, only one of the two Fab copies in the asymmetric unit was fully occupied by the peptide, likely

Next, we analyzed the bacteriophage clones by competition ELISA to identify the best scaffold for each target (pSer, pThr or pTyr) (data not shown). We identified a pSer-specific scaffold (pSAb with sequence ATGGHT), a pSer/pThr-specific scaffold (pSTAb with sequence STPRGST) and a pTyr-specific scaffold (pYAb with sequence VTYGGRK).

H2- and GS-library selections against pSer/pThr highlighted the conservation of the key anchoring residue T52A and conformational residue G54 in the loop, whereas more diversity was observed in the specificity residues (S52 and S54) (Fig. 2a). Notably, in the H2+1 libraries, we observed a strong enrichment for a Pro-Arg insertion in place of G53 and conservation of G54 in (Fig. 2b). The G54 residue occupies a region of the Ramachandran plot in which only glycine is allowed, thus suggesting that this glycine is critical for the conformational change in the loop. It is recognized by a salt bridge with K56 and a hydrophobic interaction between V52 and the phenyl ring of the pTyr. However, the phosphate group of pTyr does not occupy the phosphate-binding pocket, which is instead occupied by a water molecule (shown as red sphere). The structures demonstrate two distinct recognition sectors: a phosphoresidue-binding pocket (red box) and the peptide-binding ‘reader’ region (black box). Key CDRs L3, H2 and H3 are colored yellow, red and dark blue, respectively. Phosphopeptides are shaded magenta and the mAb light and heavy chains are shaded green and cyan, respectively. Yellow and black dashed lines indicate hydrogen bonds between the phosphoepitope and mAb scaffold.

Figure 2 Selection and characterization of pSer-, pSer/pThr- and pTyr-specific scaffolds. Competition ELISAs were used to determine the specificity of each mAb scaffold (n = 3, error bars represent s.d.). (a,b) For both pSAb (a) and pSTAb (b), no binding inhibition was observed for the unphosphorylated peptides up to 2 μM, whereas strong inhibition was observed for the phosphorylated peptides. (c) For pYAb, weak binding was observed at high concentrations of the unphosphorylated Tyr peptide, but ~20-fold less pTyr peptide was required for the same level of inhibition. ~20-fold less pTyr peptide was required for the same level of inhibition. ~20-fold less pTyr peptide was required for the same level of inhibition. ~20-fold less pTyr peptide was required for the same level of inhibition.

Next, we analyzed the bacteriophage clones by competition ELISA to identify the best scaffold for each target (pSer, pThr or pTyr) (data not shown). We identified a pSer-specific scaffold (pSAb with sequence ATGGHT), a pSer/pThr-specific scaffold (pSTAb with sequence STPRGST) and a pTyr-specific scaffold (pYAb with sequence VTYGGRK). We were unable to isolate a pThr scaffold that did not cross-react with the pSer peptide. To determine the phospho-selectivity of these scaffolds, we analyzed binding to the phosphorylated and unphosphorylated peptides by ELISA and surface plasmon resonance optical biosensors (Supplementary Fig. 2). Strikingly, we observed high affinity and selectivity for the phosphorylated peptide in all cases (Fig. 2 and Table 1).
Table 2 Summary of scFv hits versus ten new phosphopeptide targets

| Peptide       | Sequence                  | Number of unique scFvs | Number of phospho-specific scFvs | Kₐ (nM)³ |
|---------------|---------------------------|------------------------|----------------------------------|----------|
| P1: Caspase 3 (S12) | NTENSVDVSKSPIKNLKPEKII | 5                      | 0                               | n.d.     |
| P2: RIP3 (S227)   | REVLEPTPEpSLYEV          | 6                      | 2                               | 102 ± 15 (P2.A11) |
| P3: RIP3 (S199)   | LFWNKRKpASTASDYSV        | 23                     | 17                              | 250 ± 13 (P3.28) |
| P4: Smad2 (T8)    | MSSIILPEpTTPWVKRL        | 3                      | 2                               | 78 ± 14 (P4.B9)  |
| P5: CREB (S133)   | RRESSLPpSYKRLNL          | 4                      | 4                               | 151 ± 8 (P5.G10) |
| P6: HtrA2 (S12)   | RRRVRLpLGSITVEAW         | 21                     | 21                              | 2430 ± 150 (P6.C12) |
| P7: Akt1 (T308)   | KEIKDGAKTmPFT            | 0                      | 0                               | n.d.     |
| P8: Akt1 (S473)   | ERRPHFGpPSASGTAG         | 1                      | 1                               | >5000: (P8.H9)  |
| P9: PKCδ (S695)   | DGNMFRNPpSFMPNGMER       | 1                      | 0                               | n.d.     |
| P10: Sgk1 (S422)  | AEAAEFLGpP8YAPXDF5       | 4                      | 4                               | 42.2 ± 2.8 (P10.D6) |

supplementary scFv clones that exhibited a more than fivefold higher ELISA signal against phosphorylated peptide compared to unphosphorylated peptide (Fig. 4). PAs determined by competition ELISA with scFv-Fc protein (n = 2–3, error values represent s.d.). Clone ID is shown in parentheses. Only partial competition was observed at the concentrations of peptide used. Phosphoresidue is shown in bold.

due to the packing arrangement of the Fabs (Supplementary Fig. 3).

No changes in the positions of the CDRs were observed between the mouse⁹ and humanized mAbs (cα root mean squared deviation (r.m.s.d.) of 0.78 Å). Furthermore, binding of the peptide to the mAb did not induce any major CDR movements (cα r.m.s.d. of 1.3 Å) (Supplementary Fig. 4). For all phosphopeptides, the recognition is achieved through two sectors: the phosphoresidue-binding pocket and a neighboring peptide sequence ‘reader’ region, which consists primarily of CDRs L3 and H3 (Fig. 3e). Additionally, all peptide:Fab contacts outside of the phosphoresidue also occur in the parent Fab (Supplementary Fig. 4c).

Structures of the peptide:Fab complexes illustrate how CDR H2 specifically recognizes each phosphoresidue (Fig. 3). For all three scaffolds, mutations found in the parent H2 loop make the main chain more accessible, creating a large electrostatics binding pocket (Supplementary Fig. 5). The phosphoresidue side chain is almost fully engulfed by the mAb in pSAb (80% buried) and pSTAb (92% buried) and anchored by multiple hydrogen bonds (Fig. 3a–c, and Supplementary Table 5). In pSAb, the pSer residue makes key contacts with specificity residues G53H, R55H and T56H, whereas in pSTAb, the scFv clones that exhibited a more than fivefold higher ELISA signal against phosphorylated peptide compared to unphosphorylated peptide were isolated (Table 2). For all three rounds of selection and analyzed single phage clones from the third round of selection by single-point ELISA. For seven targets, we isolated at least one scFv that bound only to the phosphorylated antigen (Table 2 and Fig. 4a). To test the specificity of the isolated clones, we performed a panel of ELISAs to assay binding of each scFv to each of the ten phosphorylated peptides (Fig. 4b). The data demonstrates the exquisite target selectivity of most scFv clones, indicating the absence of promiscuous pSer-pThr-peptide binding scFvs. Western blot analysis confirmed that a sample set of mAbs specifically recognized the corresponding phosphoprotein (Fig. 4c).

Finally, the scFv-Fc fusions exhibited affinities ranging from 42 to 919 nM. Notably, the H2 nest pocket is occupied by a water molecule that is stabilized by the free C terminus of the peptide, indicating that pYAb may bind differently to the pTyr residue in longer peptides without this neighboring free carboxylate (Fig. 3d). Combined, our in vitro characterization and X-ray crystal structures confirmed that we successfully designed mAbs that use pSer or pThr as hot-spot residues.

Generation of target- and phospho-specific mAbs using the pSer and pSer/ pThr scaffolds

We hypothesized that an antibody library in which the phosphoresidue-binding pocket was conserved and ‘reader’ regions were mutated would enable rapid generation of new target- and phospho-specific mAbs. Because every member of the initial library contains a phosphoresidue-binding pocket, each mAb should have a weak initial affinity for the phosphorylated antigen, dramatically enhancing the selection of new mAbs. In proof-of-principle experiments, we targeted antigens containing pSer and pThr, as reagents capable of detecting these modifications are rare. We diversified surface-exposed positions in CDR H2 (50HH, 50HT, 50SH) outside of the phosphate-binding pocket, CDR H3 (95HH–101HH), and CDR L3 (91L–94L, 96L) (Supplementary Table 6). We chose a set of ten biologically relevant pSer- or pThr-containing epitopes as target antigens (Table 2). To increase the stringency of our test, we did not perform counter-selections against the unphosphorylated antigens because we reasoned that the binding pocket could be sufficient for the selection of mAbs that required the phosphorylated residue. We performed three rounds of selection and analyzed single phage clones from the third round of selection by single-point ELISA.

Figure 4 Generation of recombinant phospho-specific (PS) mAbs using the pSAb and pSTAb scaffolds. (a) Representative phage ELISAs of one scFv clone selected against each of the nine phosphopeptide targets demonstrates that we selected phospho-specific mAbs to seven out of the ten targets. No hits were observed against P7. To analyze target specificity, we characterized the binding of each scFv-phage to ten different phosphopeptides by phage ELISA (n = 2–3). (b) Heatmap representation of the phage ELISA binding signals for each scFv-phage (horizontal axis) against each of the ten phosphopeptides (vertical axis). Strikingly, most of these scFvs bind only to the phosphopeptide against which they were selected. For each scFv, signals were normalized to the highest overall ELISA signal observed against the ten peptides. The scale goes from zero (black) to one (yellow). (c) scFvs also recognize the phosphorylated protein in western blots. FLAG-tagged target proteins were immunoprecipitated from transiently transfected HEK293T cells. To verify phospho-specific binding, samples were either dephosphorylated using alkaline phosphatase (AP) or treated with buffer only. Membranes were probed with biotinylated scFv (20 μg/ml) overnight and bound scFv was detected using NeutrAvidin-HRP. Total levels of target protein were monitored using anti-FLAG-HRP.
~5,000 nM (Table 2), which matches or exceeds previous reports of phospho-specific mAb affinities.

**DISCUSSION**

Here we describe a recombinant mAb-generation method that entails the design of a motif-specific (for example, pSer, pThr or pTyr) mAb scaffold followed by structure-informed mutagenesis of the scaffold to generate mAbs against a panel of phosphopeptide antigens. The high success rate of our strategy (phospho-specific mAbs against seven of ten targets), which does not employ counter-selections against the unphosphorylated epitope, demonstrates how the motif-specific pocket greatly improves the selection process, as even past antibody libraries generated from immunized animals required stringent counter-selections to enrich for phospho-specific mAbs. In the case of pSAb and pSTAb, the pocket contains a hallmark α7 glycine at 54%H that contributes to the main-chain conformation of CDR H2. There is a very high frequency of occurrence for this H2 conformation in mAbs (~12% of all H2 conformations), and multiple mAb structures with anionic molecules (for example, aspartate, glutamate or sulfate) bound at this site (Supplementary Fig. 1).

While our studies were in progress, the structure of a chicken scFv, which was generated from an immunized phage display library, was reported. The antibody was found to have a similar H2 conformation that bound the pThr-containing phosphopeptide. Notably, a structural comparison of this chicken scFv with our mAbs reveals that the phosphoresidue binds to the same H2 loop conformation albeit with a different hydrogen bonding pattern (Supplementary Fig. 6). This striking similarity suggests there may be a germline-encoded, anion-binding pocket capable of binding phosphate or sulfate groups. In fact, previous work on mAbs that bind phospholipids suggested a ‘phosphate-binding subsite’ that conferred recognition of only the phosphorylated or sulfated forms of multiple lipids and haptens. Furthermore, anion-binding, pocket-containing mAbs may provide a protective role in the recognition of phosphorylated or sulfated antigens, such as lipid A in Gram-negative bacteria, or conversely, a more sinister role in autoimmune diseases, such as antiphospholipid syndrome. Future crystallographic studies of these mAb:antigen complexes will illuminate this intriguing possibility.

Interestingly, the main-chain dominated mode of pSer/pThr recognition is completely different from most endogenous pSAb/pTThr-binding domains such as SH2, 14-3-3 and FHA, which predominantly use side chains to bind the phosphoresidue (Fig. 3 and Supplementary Fig. 7). Only the WW domain sometimes uses two main-chain amides to bind a phosphate. In fact, our pSer/pThr scaffolds bind more efficiently to the phosphoresidue than naturally occurring domains by burying a larger surface area and contributing more hydrogen bonds (Supplementary Table 5). It was recently suggested that these endogenous phosphoresidue-binding and other PTM-binding domains have evolved to bind shorter epitopes with moderate affinities to support the dynamic nature of signal transduction pathways, which potentially limits the range of epitopes they can bind. Additionally, our designed phospho-specific pockets appear to function independently of the other CDRs as we could diversify those CDRs to target highly diverse phosphopeptides (Fig. 4).

Surprisingly, pYAb uses a completely different motif to recognize pTyr. It is notable that we achieved highly specific recognition of pTyr, despite not burying most of the pTyr phenyl ring (Figs. 2c and 3d). However, we have yet to determine how the presence of the free carboxylate, which stabilizes a water molecule in the nest, contributes to the binding affinity. We are currently developing new scaffolds in which most of the pTyr residue is buried and bound in a more nest-like region to boost the ligand efficiency and affinity.

Our bacteriophage-derived phospho-specific mAb platform, which can be automated, rapidly generates mAbs within 2 weeks as opposed to more than several months required for hybridoma methods. In stark contrast to traditional monoclonal or polyclonal phospho-specific antibodies, our recombinant phospho-specific mAbs use a single framework that permits high-level bacterial expression (>3 mg/l) and mammalian expression (~0.5–5 µg/ml media) in a renewable format. The use of a single framework greatly simplifies mutagenesis protocols (for example, affinity maturation), sequence-function analysis and conversion to other mAb formats (for example, IgG). Finally, we hypothesize that this motif-specific scaffold method should be generalizable to targeting virtually any antigen with a defined motif. As many other PTM-binding motifs exist in nature, these motifs may be similarly designed into mAbs to generate high-affinity monoclonal reagents capable of detecting other PTMs. Ultimately, the rapid in vitro generation of monoclonal anti-PTM antibodies will greatly enhance the study of PTMs throughout biology.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** PDB: X-ray coordinates for pSAb:pSer (4JFZ), pSTAb:pSer (4JG0), pSTAb:pThr (4JG1), pYAb:pTyr (4FX) and pYAb (4FY).

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank members of the Wells laboratory for helpful discussions regarding this manuscript and S. Pfaff for assistance with Biacore experiments. We thank C. Wadding at the UCSF X-ray facility for assistance with generating protein crystals and J. Holton, G. Meigs and J. Tanamachi at the Advanced Light Source beam line 8.3.1 at the Lawrence Berkeley National Laboratory for help with collection of diffraction data. We thank the Court laboratory at the National Institutes of Health for generously providing the recombineering vectors. J.T.K. is a Fellow of the Life Sciences Research Foundation and N.D.T. is the Suzanne and Bob Wright Fellow of the Damon Runyon Cancer Research Foundation. This work was supported by grants from the US National Institutes of Health (R01 CA154802 to J.A.W. and GM04616 to WED). J.T.K., J.A.W. and WED have filed a provisional patent on the technology described in this manuscript.

**AUTHOR CONTRIBUTIONS**

J.T.K. designed and executed experiments; N.D.T. assisted with crystallography. B.T.H. and W.F.D. assisted with modeling experiments; J.A.W. designed and supervised experiments. J.T.K. and J.A.W. wrote the manuscript with input from all co-authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Cohen, P. The regulation of protein function by multisite phosphorylation—a 25 year update. Trends Biochem. Sci. 25, 596–601 (2000).
2. Haniuhan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. Cell 144, 646–674 (2011).
3. Blagoev, B., Ong, S.E., Kratchmarova, I. & Mann, M. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat. Biotechnol. 22, 1139–1145 (2004).
4. Zhou, H., Watts, J.D. & Aebersold, R. A systematic approach to the analysis of protein phosphorylation. Nat. Biotechnol. 19, 375–378 (2001).
5. Hornbeck, P.V., Chabra, I., Kornhauser, J.M., Skrzypek, E. & Zhang, B. PhosphoSite: A bioinformatics resource dedicated to physiological protein phosphorylation. *Proteomics* 4, 1551–1561 (2004).

6. Beausoleil, S.A. et al. Large-scale characterization of HeLa cell nuclear phosphophosphoproteins. *Proc. Natl. Acad. Sci. USA* 101, 12130–12135 (2004).

7. Bendall, S.C. et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* 332, 687–696 (2011).

8. Sachs, K., Perez, O., Pe'er, D., Lauffenburger, D.A. & Nolan, G.P. Causal protein-signaling networks derived from multiparameter single-cell data. *Science* 308, 523–529 (2005).

9. Brumbaugh, K. et al. Overview of the generation, validation, and application of phosphosite-specific antibodies. *Methods Mol. Biol.* 717, 3–43 (2011).

10. Dopfer, E.P. et al. Analysis of novel phospho-ITAM specific antibodies in a T2 reconstitution system for TCR-CD3 signaling. *Immunol. Lett.* 130, 43–50 (2010).

11. DiGiovanna, M.P. & Stern, D.F. Activation state-specific monoclonal antibody detects tyrosine phosphorylated p185neu/erbB-2 in a subset of human breast tumors overexpressing this receptor. *Cancer Res.* 55, 1946–1955 (1995).

12. Nita-Lazar, A., Saito-Benz, H. & White, F.M. Quantitative phosphoproteomics by mass spectrometry: past, present, and future. *Proteomics* 8, 4433–4443 (2008).

13. Marks, J.D. et al. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222, 581–597 (1991).

14. McCafferty, J., Griffiths, A.D., Winter, G. & Chiswell, D.J. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552–554 (1990).

15. Kang, A.S., Barbas, C.F., Janda, K.D., Benkovic, S.J. & Lerner, R.A. Linkage of recognition and replication functions by assembling combinatorial antibody Fab filamentous phage displaying antibody variable domains. *Science* 267, 383–386 (1995).

16. Clackson, T. & Wells, J.A. A hot spot of binding energy in a hormone-receptor interface. *Science* 267, 1287–1292 (2000).

17. Sidhu, S.S. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222, 581–597 (1991).

18. Mersmann, M. et al. Towards proteome scale antibody selections using phage display. *Nat Biotechnol.* 27, 118–128 (2010).

19. Feldhaus, M.J. et al. Flow-cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library. *Nat. Biotechnol.* 21, 163–170 (2003).

20. Cobaugh, C.W., Almagro, J.C., Pagson, M., Iverson, B. & Georgiou, G. Synthetic antibody libraries focused towards peptide ligands. *J. Mol. Biol.* 378, 622–633 (2008).

21. Shih, H.H. et al. An ultra-specific avian antibody to phosphorylated tau protein reveals a unique mechanism for phosphoepitope recognition. *J. Biol. Chem.* 287, 44425–44434 (2012).

22. Vliegelaer, D. et al. Direct selection of monoclonal phosphospecific antibodies without prior phosphoamino acid mapping. *J. Biol. Chem.* 284, 20791–20795 (2009).

23. Kaneko, T. et al. Superbinder SH2 domains act as antagonists of cell signaling. *Sci. Signal.* 5, ra68 (2012).

24. Peshad, K., Wyssinian, K. & Kay, B.K. Directed evolution of the forkhead-associated domain to generate anti-phosphospecific reagents by phage display. *J. Mol. Biol.* 424, 88–103 (2012).

25. Malabarba, M.G. et al. A repertoire library that allows the selection of synthetic SH2s with altered binding specificities. *Oncoogene* 20, 5186–5194 (2001).

26. Watson, J.D. & Milner-White, E.J. A novel main-chain anion-binding site in proteins: the nest. A particular combination of phi,psi values in successive residues gives rise to anion-binding sites that occur commonly and are found often at functionally important regions. *J. Mol. Biol.* 315, 171–182 (2002).

27. Landry, R.C. et al. Antibody recognition of a conformational epitope in a peptide antigen: Fv-peptide complex of an antibody fragment specific for the mutant EGF receptor, EGFRvIII. *J. Mol. Biol.* 308, 883–893 (2001).

28. Hollingsworth, S.A. & Karplus, P.A. A fresh look at the Ramachandran plot and the occurrence of standard structures in proteins. *Biomed Concepts* 1, 271–283 (2010).

29. North, B., Lehmann, A. & Dunbrack, R.L. Jr. A new clustering of antibody CDR loop conformations. *J. Mol. Biol.* 406, 228–256 (2011).

30. Levine, J.S., Branch, D.W. & Rauch, J. The antiphospholipid syndrome. *N. Engl. J. Med.* 346, 752–763 (2002).

31. Alving, C.R. Antibodies to liposomes, phospholipids and phosphate esters. *Phys. Lipids* 40, 303–314 (1986).

32. Kaneko, T., Joshi, R., Feller, S.M. & Li, S.S. Phosphotyrosine recognition domains: the typical, the atypical and the versatile. *Cell Commun. Signal.* 10, 32 (2012).

33. Seet, B.T., Dikic, I., Zhou, M.M. & Pawson, T. Reading protein modifications with interaction domains. *Nat. Rev. Mol. Cell Biol.* 7, 473–483 (2006).
ONLINE METHODS

Vector construction. We constructed a series of pJ phage display vectors along with compatible protein expression vectors (Supplementary Table 2). We modified the human Fab template by Kunkel mutagenesis, according to standard protocols. All restriction enzymes and DNA polymerases were purchased from NEB (Ipswich, MA). Oligonucleotides were purchased from IDT and all constructs were verified by DNA sequencing (Quintara Biosciences).

Generation of phage libraries. A humanized Fab in pJK1 with two stop codons within the CDR H2 was used as a template for Kunkel mutagenesis with oligonucleotides designed to correct the stop codons and introduce the designed mutations at each site. To make the H2-targeted libraries, we generated three libraries in which the codons encoding for the parent H2 sequence (STGGYN) was replaced with either (i) six random amino acids encoded by NNK (H2 library), (ii) seven random amino acids encoded by NNK (H2+1 library), or (iii) a core set of two or three amino acids, which were allowed to be only Gly or Ser, and were flanked on both sides by two random amino acids encoded by NNK (GS library). Mutagenic oligonucleotides are listed in Supplementary Table 7. The resulting mutagenesis reactions were electrophorated and phage were produced as previously described. The final diversities of the H2, H2+1 and GS libraries were 6.5 × 10^6, 1.6 × 10^7 and 5.3 × 10^7, respectively.

To make the phage-specific antibody libraries, we constructed two scFv templates, which consisted of either the pSAb or pSTAb variable light chain linked to the corresponding variable heavy chain by a (GlySer)n linker and contained two stop codons in the CDR H3. These plasmids were then used as templates for Kunkel mutagenesis. The light chain CDR L3 (91L-94L, 96L) and the heavy chain CDR H2 (50H, 56H, 58H) were diversified using degenerate codons designed to mimic the natural sequence diversity found at these positions (Supplementary Table 6). CDR H3 was diversified using three to nine random amino acids (DVK) followed by three terminal residues (F/M, A/D and Y) commonly observed in anti-peptide mAbs. For the mutagenesis reactions, L3 oligonucleotides (P1 and P2) were mixed at a 1:1 molar ratio, whereas L3 oligonucleotides (P1 and P2) were mixed at a 1:1:2 ratio and H3 oligonucleotides (PX.1 and PX.2, where X = CDR length) were mixed at a 2:1 ratio. The resulting libraries were produced using Hyperphage to enhance recovery of rare binders and the final diversities of the pSAb and pSTAb libraries were 3.4 × 10^10 and 2.7 × 10^10, respectively.

Phage display selections, ELISAs and western blot analysis. All phage preparations and ELISAs were done according to standard protocols. Briefly, 96-well Maxisorp plates were coated with 10 µg/ml NeutrAvidin overnight at 4 °C and subsequently blocked with 2% BSA for 2 h at 20 °C. Various concentrations of Fab-phage were mixed with a fixed concentration of biotinylated peptide and captured on the NeutrAvidin-coated wells for 15 min. The bound phage were then detected using a horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal (GE Healthcare). For phage competition ELISAs, plates were coated with 10 µg/ml NusA-AGNYVVTDH (the native target for the 1i8i Fab and a weak binder to pSAb, pSTAb and pYab) and blocked with 2% BSA. Subsaturating levels of phage were then pre-bound to the various concentrations of peptide antigens for 2 h at 20 °C and then captured on the NusA-peptide coated plates for 15 min. For scFv-Fc competition ELISAs, plates were coated with NeutrAvidin, blocked with 2% BSA, incubated with 100–200 nM biotinylated peptide, and finally blocked with 200 µM biotin. scFv-Fcs were then pre-bound to a dilution series of peptide antigen and processed as described for phage. Bound scFv-Fcs were detected with HRP-conjugated Protein A (Pierce). Competition ELISA data were fit using a four-parameter logistic equation, with error shown by s.d. of 2–3 replicates for each sample analyzed. Selections with the H2-targeted libraries were performed using biotinylated phosphopeptide antigens captured with streptavidin-coated magnetic beads (Promega). In total, four rounds of selection were performed with decreasing amounts of peptide antigen (500, 250, 100 and 10 nM) and individual phage clones were analyzed from the fourth round of selection. Selections with the pSAb and pSTAb libraries were identically performed except only three rounds were conducted.

For western blot analysis, HEK293 cells were transiently transfected with a pcDNA3.1 vector that expresses the cDNA encoding for each protein fused to a 3xFLAG-V5 epitope tag. CREB-transfected cells were stimulated with 10 µM forskolin for 30 min. Akt1- or Sgk1-transfected cells were stimulated by growth in 10% FBS for 24 h. Cells were lysed and the target protein was immunoprecipitated using anti-FLAG M2 agarose (Sigma-Aldrich). To dephosphorylate each protein, each immunoprecipitated sample was split and either treated with alkaline phosphatase or buffer only. Samples were separated on a 4–12% SDS-PAGE gel and transferred to PVDF membrane. The membrane was then blocked with 5% BSA and incubated overnight with biotinylated scFv (20 µg/ml) overnight at 4 °C. The membranes were then washed and incubated for 1 h with NeutrAvidin-HRP (1:5,000) (Pierce). Immunoreactivity was detected using chemiluminescence. The amount of total protein was assessed by reprobing with anti-FLAG-HRP antibody (1:1,000; Sigma-Aldrich).

Protein expression and purification. Selected Fabs were expressed in a protease-deficient C43 strain. Expressed Fabs were purified from total cell lysates by Protein A, ion exchange and gel filtration chromatography, as previously described. Fabs were stored at 4 °C for short-term analysis or flash frozen in 10% glycerol for storage at −80 °C. ScFv-Fc constructs were transiently expressed into 293T cells and purified from the media using Protein A chromatography. Biotinylated scFvs contained a C-terminal biotin-acceptor peptide and were co-expressed with BirA to enzymatically biotinylate each protein (pJK5). Nonphosphorylated versions of all peptides were fused to the C terminus of NusA, which contains an N-terminal His6 tag and biotin acceptor peptide. Recombinant proteins were purified on a His GraviTrap column (GE Healthcare, Piscataway, NJ) followed by monomeric Avidin resin (Thermo Scientific, Rockford, IL) to a final purity of >95%. All biotinylated peptides were purchased from Elix Biopharmaceuticals (Hayward, CA) or Pepthody, Inc. (Charlotte, NC).

Biacore analysis. Surface plasmon resonance data were measured on a Biacore model 4000 (Biacore, Uppsala, Sweden). All proteins were in TBS containing 0.1 mg/ml BSA and 0.01% Tween-20. A Biacore CMS chip was coated with NeutrAvidin at ~3,000 RU and biotinylated antigens were captured at <100 RU. Serial dilutions of the Fabs were flowed over the immobilized antigens and 1:1 Langmuir binding models were used to calculate the k_on, k_off and K_D for each Fab:antigen pair.

Crystallization of Fab:peptide complexes. Fabs were expressed as described above and concentrated to 10–15 mg/ml in 10 mM Tris pH 7.5, 50 mM NaCl. Complexes of the Fab with the corresponding peptide were formed at a 1:2 molar ratio of Fab:peptide. Crystals were grown in hanging drop format by mixing 100 nl protein solution and 100 nl crystallization solution using a Mosquito nanoliter pipetting system (TTP Labtech). Crystals formed within 1–2 weeks at 18 °C or 4 °C. Initially, the crystals we obtained for the Fabs bound to the pSer peptides diffracted very weakly. We therefore employed a microseeding strategy with a seed stock generated from finely ground pSTAb:pThr crystals in 50 µl cryoprotectant solution. Crystals for the pSAb:pSer and pSTAb:pSer complexes were generated by hanging drop vapor diffusion with 300 nl drops consisting of 150 µl protein solution, 120 nl reservoir solution, and 30 nl 1:10 dilution of seed stock. All crystals were soaked in cryoprotectant solution and flash frozen in liquid nitrogen. Crystallization conditions and cryoprotectant solutions are listed in Supplementary Table 3.

Diffraction data were collected using the Advanced Light Source beam line 8.3.1 at the Lawrence Berkeley National Laboratory (Berkeley, California) with a wavelength of 1.1 Å. The data were indexed, integrated and scaled using ELVES® or HKL2000 (ref. 43). The structure of the pSTAb:pThr complex was solved by molecular replacement using Phenix. The initial search model consisted of the variable heavy domain from 3n9g and the variable light domain, constant heavy domain, and constant light domain from 2gcy5. The pSTAb Fab structure was used as the search model for all other structures. Iterative rounds of model building and refinement were carried out with Phenix and Coot. For isomorphous crystals, the same refinement test sets for calculating Rfree were used. Simulated annealing composite omit maps calculated using Phenix were used to remove model bias. After two rounds of refinement, peptides were built into each model using Coot. Riding hydrogens as implemented in Phenix were used in the final stages of refinement for the pSAb:pSer, pSTAb:pSer and pSTAb:pThr complexes. Final refinement statistics can be found in Supplementary Table 4. The final coordinates were validated using MolProbity. The final Ramachandran statistics (% Favored; % Outlier) were

© 2013 Nature America, Inc. All rights reserved.
98:0.2, 98:0.2, 98:0.2, 98:0 and 97:0.2 for pSAb:pSer, pSTAb:pSer, pSTAb:pThr, pYAb:pTyr and pYAb, respectively. MacPyMol (DeLano Scientific) was used to generate structure figures. Electrostatic surfaces were calculated using APBS48 and buried surface areas were calculated using CCP4 (ref. 49).

37. Kunkel, T.A. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488–492 (1985).
38. Bostrom, J. et al. Variants of the antibody herceptin that interact with HER2 and VEGF at the antigen binding site. Science 323, 1610–1614 (2009).
39. Rondot, S., Koch, J., Breitling, F. & Dubel, S. A helper phage to improve single-chain antibody presentation in phage display. Nat. Biotechnol. 19, 75–78 (2001).
40. Thomsen, N.D., Koerber, J.T. & Wells, J.A. Structural snapshots reveal distinct mechanisms of procaspase-3 and -7 activation. Proc. Natl. Acad. Sci. USA 110, 8477–8482 (2013).
41. Luft, J.R. & DeTitta, G.T. A method to produce microseed stock for use in the crystallization of biological macromolecules. Acta Crystallogr. D Biol. Crystallogr. 55, 988–993 (1999).
42. Holton, J. & Alber, T. Automated protein crystal structure determination using ELVES. Proc. Natl. Acad. Sci. USA 101, 1537–1542 (2004).
43. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).
44. Adams, P.D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 (2010).
45. Kaufmann, B. et al. Neutralization of West Nile virus by cross-linking of its surface proteins with Fab fragments of the human monoclonal antibody CR4354. Proc. Natl. Acad. Sci. USA 107, 18950–18955 (2010).
46. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132 (2004).
47. Chen, V.B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D Biol. Crystallogr. 66, 12–21 (2010).
48. Baker, N.A., Sept, D., Joseph, S., Holst, M.J. & McCammon, J.A. Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. USA 98, 10037–10041 (2001).
49. Winn, M.D. et al. Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 67, 235–242 (2011).