A Robust Protocol to Map Binding Sites of the 14-3-3 Interactome: Cdc25C Requires Phosphorylation of Both S216 and S263 to bind 14-3-3

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Modern proteomic techniques have identified hundreds of proteins that bind 14-3-3s, the most widespread eukaryotic phosphoserine/threonine sensors, but accurate prediction of the target phospho-sites is difficult. Here we describe a systematic approach using synthetic peptides that tests large numbers of potential binding sites in parallel for human 14-3-3. By profiling the sequence requirements for three diverse 14-3-3 binding sites (from IRS-1, IRSp53 and GIT2), we have generated enhanced bioinformatics tools to score sites and allow more tractable testing by co-immunoprecipitation. This approach has allowed us to identify two additional sites other than Ser216 in the widely studied cell division cycle (Cdc) protein 25C, whose function depends on 14-3-3 binding. These Ser247 and Ser263 sites in human Cdc25C, which were not predicted by the existing Scansite search, are conserved across species and flank the nuclear localization region. Furthermore, we found strong interactions between 14-3-3 and peptides with the sequence Rxx[S/T]xR typical for PKC sites, and which is as abundant as the canonical Rxx[S/T]xP motif in the proteome. Two such sites are required for 14-3-3 binding in the polarity protein Numb. A recent survey of >200 reported sites identified only a handful containing this motif, suggesting that it is currently under-appreciated as a candidate binding site. This approach allows one to rapidly map 14-3-3 binding sites and has revealed alternate motifs.

It is more than four decades since the isolation of 14-3-3 proteins from brain extracts (1) followed by their characterization as regulators of phosphoserine and threonine bearing proteins (2). They have broad significance in biological signaling as protein phosphorylation predominantly occurs on serine/threonine residues in vivo (3). The roles of 14-3-3s in cellular behavior such as proliferation and survival, and their links to disease states such as cancers and neuropathologies are well reviewed (4, 5); therefore 14-3-3s can be regarded as generic transducers of phosphoserine and threonine signaling in physiological and aberrant settings. They are predominantly dimers with the binding pocket accommodating ~7–10 residues (6). By coupling to two phospho-sites 14-3-3s can constrain the protein conformation, compete for binding with other proteins, or bridge different phosphoproteins (though not yet formally demonstrated) (7, 8).

The handful of 14-3-3 targets described in the early 1990s (9) has swelled to current estimates of >500 based on proteomic affinity-based studies (8, 10–14). 14-3-3 likely plays a key role in the function of each target: thus mapping the interacting phospho-sites often provides mechanistic insights. However, the most arduous part of protein: protein interaction studies is often the mapping analysis because it involves labor-intensive methods. Mapping analyses are further complicated in proteomic studies because direct and indirect binders are difficult to distinguish in protein complexes. The modest overlap (~25%) among the lists of 14-3-3 associated partners generated by the proteomic studies above (8) suggests that alternate targets are identified in different experimental settings. This has resulted in a discrepancy in which members of the 14-3-3 interactome have increased at a rapid pace but site identification has not kept up. For these reasons, in vitro assays employing synthetic peptides with full phosphorylation and well-defined ligand binding conditions can help to overcome these limitations. A pioneering study of 14-3-3 specificity using soluble peptide libraries (15) defined binding motifs currently used as consensus sequences, with Pro strongly represented two positions carboxyl to the phospho-residue (Pro₋₋), currently used by the Scansite search engine (16). The early studies had to contend with inherent limitations of older technologies: unequal representation of peptides in a pool, and sequence identification had the obstacles of decreasing cycle yields, variability in amino acid cleavage, and cycle carry-over (17, 18). A recent survey of 201 mammalian 14-3-3 binding sites indicates that only half contained Pro₋₋₋.
14-3-3 Site Mapping Using Bioinformatics and Peptide Arrays

Consensus motifs for 14-3-3 binding sites can fail to correctly identify sites within established 14-3-3 interactors (19), suggesting that 14-3-3 target selectivity is not fully established and needs to be updated.

We describe here an approach that uses array-based peptides to generate new Scansite-format matrices, which in turn are used to predict binding sites in known interactors. A range of potential sites can be pared down by in situ validation using the same protocol, followed by standard mutagenesis and co-immunoprecipitation. This protocol can rapidly extend and refine site coverage for human 14-3-3 and provides evidence for sequences not previously appreciated in the literature.

MATERIALS AND METHODS

Purification of Recombinant 14-3-3—Human 14-3-3 isoforms were cloned into a modified pGEX (GE Healthcare Life Sciences) bicistronic vector pGEX-4T-BiotinN containing a 10-residue biotin acceptor sequence amino-terminal to the start codon of the insert and the BirA gene. In these constructs the 10-residue biotin acceptor sequence was engineered in binding buffer (20 mM Hepes pH 7.3, 137 mM NaCl, 5 mM KCl, 0.05% Tween-20) and downstream 14-3-3 insert (20). The resulting pGEX-BiotinN-14-3-3 constructs were transformed into BL21(DE3) E. coli strain for expression. GST-biotin-14-3-3 constructs were purified using glutathione-agarose and desalted using PD-10 (GE Healthcare) gel filtration columns. All isoforms were confirmed to be similarly biotinylated by streptavidin-horseradish peroxidase (HRP) overlay (data not shown). His6 tagged 14-3-3 was expressed using pQE-30 expression vector (Qiagen, Hilden, Germany) and purified using Protein Ni-TED 2000 packed columns (Macherey-Nagel, Bethlehem, PA). Recombinant protein purity was estimated to be >95% by Coomassie staining (supplementary Fig. S1).

14-3-3 Overlay Assays Using Peptide Arrays—Individual peptides were synthesized using standard chemistry in situ (PepSpots) on cellulose based matrix (Jenri Biotechos, Berlin, Germany). To ensure protein accessibility, each 11-residue peptide (N-terminal acetylated) contained a four-residue spacer consisting of a glycine and three β-alanines. All peptides were immobilized via the C terminus. To assess synthesis reproducibility, the parental sequences of the three substituited peptide sequences (Fig. 2 and supplementary Fig. S2) and the nonphosphorylated forms were confirmed to be triplicate in different rows on each array. Standard error values are indicated above the bar corresponding to the parental sequence residue. Prior to usage, peptide array membranes were washed in binding buffer (20 mM Hepes pH 7.3, 137 mM NaCl, 5 mM KCl, 0.05% Tween-20) and blocked with 5% filtered bovine serum albumin in the same buffer, Recombinant biotinylated 14-3-3 peptides were diluted to 10 μg/ml in binding buffer, and incubated for 30 min at room temperature. The filters were washed (10 min × 2) and streptavidin-HRP (1:20,000, GE) and incubated for 15 min at room temperature, then washed in binding buffer (3 × 10 min). Bound 14-3-3 was revealed using enhanced chemiluminescence and standard x-ray film: no signal was detected for nonphosphorylated sequences (Fig. 1 and supplementary Fig. S2) and the nonphosphorylated forms were confirmed to be triplicate in different rows on each array. Standard error values are indicated above the bar corresponding to the parental sequence residue. Prior to usage, peptide array membranes were washed in binding buffer (20 mM Hepes pH 7.3, 137 mM NaCl, 5 mM KCl, 0.05% Tween-20) and blocked with 5% filtered bovine serum albumin in the same buffer. Recombinant biotinylated 14-3-3 peptides were diluted to 10 μg/ml in binding buffer, and incubated for 30 min at room temperature. The filters were washed (10 min × 2) and streptavidin-HRP (1:20,000, GE) and incubated for 15 min at room temperature, then washed in binding buffer (3 × 10 min). Bound 14-3-3 was revealed using enhanced chemiluminescence and standard x-ray film: no signal was detected for nonphosphorylated sequences (Fig. 1) and no background was detected on these blots with streptavidin-HRP alone (data not shown).

1 The abbreviations used are: Cdc25, cell division cycle 25; Cdc42, cell division cycle 42; IRSp53, insulin receptor substrate protein of 53 kDa; GIT, GPCR kinase interacting protein; SPR, surface plasmon resonance; HRP, horseradish peroxidase; SBP, streptavidin binding protein; PKC, protein kinase C.
**Fig. 1. The use of phospho-peptide arrays to detect 14-3-3 target sequences.** A. Schematic diagram of the 14-3-3 overlay procedure. Synthetic peptides immobilized via their C termini were probed using biotinylated 14-3-3 and detected by streptavidin-HRP chemiluminescence. Blue spheres represent variant peptide residues for testing with N-terminal acetylation denoted by “Ac” and green spheres represent invariant 14-3-3 Site Mapping Using Bioinformatics and Peptide Arrays.

**Table:**

| Spot | Protein ID | Sequence   | Interaction Strength |
|------|------------|------------|---------------------|
| 1    | S259       | RQRST[pS]TPSVH | + + + +              |
| 2    | S621       | LRRE[pS]VSQDI | + + + +              |
| 3    | S297       | LRXX[pS]HEQOL | + + + +              |
| 4    | S125       | INKKL[pS]LPADI | +++ +++               |
| 5    | S146       | LNKKL[pS]LPADI | +++ +++               |
| 6    | S112       | RRRB[pS]YPAGT | + + + +              |
| 7    | S136       | RRRB[pS]RPRLN | +++ +++               |
| 8    | S309       | LFRSP[pS]MPCSV | - -                 |
| 9    | S309       | LFRSP[pS]LPCSV | - -                 |
| 10   | S309       | LFRSP[pS]LPCSV | - -                 |
| 11   | S310       | AQRH[pT]LPASH | ++ +++               |
| 12   | S885       | DPRH[pS]LPAGD | ++ +++               |
| 13   | S39        | PHLRF[pS]MPVDR | - +                |
| 14   | S22        | PQRR[pS]PAPA   | + + + +              |
| 15   | S937       | LRRS[pS]SDSH   | - -                 |
| 16   | S978       | LRRS[pS]LAHQL | + ++                 |
| 17   | S374       | LNHSR[pS]IPMPA | + +                 |
| 18   | S641       | SFKV[pS]IAPQQQ | + +                 |
| 19   | S167       | YLR[S]IP[pS]VFV | + +                 |
| 20   | S259       | LRXTA[pS]EPNLK | + +                 |

**IRSp53 Sites:**

- **Human:** DSYSNTLPRKSVPKNSYATENKTTPRSS
- **Mouse:** DSYSNTLPRKSVPKNSYATENKTTPRSS
- **Zebrafish:** DYSNTLPRKSPPAKNKTVGERTLTPRSS
- **Frog:** DSYSNTLPRKSVPPVSYTAENKTTPRSS

**Spot #**

- **14-3-3η:**
  - 1: 20 secs
  - 21: 1 min
- **14-3-3ζ:**
  - 20: 20 secs
  - 40: 1 min

**Interaction Strength:** + = weak, ++ = moderate, +++ = strong, - = not detected.
Streptavidin-HRP blotting confirmed minimal levels of endogenous biotinylated proteins in the final eluate (data not shown).

RESULTS

A phosphopeptide Overlay Protocol Can Assess 14-3-3 Binding—We reasoned that bivalent 14-3-3 might bind tightly to immobilized peptides at sufficiently high density on arrays, and tested the overlay protocol illustrated in Fig. 2. A test set of synthetic phosphopeptides derived from validated mammalian targets for 14-3-3 (references in supplementary Table S1A), included two new sites T340 and T360 in the cell division cycle 42 (Cdc42) effector insulin receptor substrate protein of 53 kDa (IRSp53) (23). These do not contain basic residues of the linker sequence.

Yellow spheres correspond to the phosphate moiety of the Ser/Thr residue. Clear spheres labeled with “b” represent the biotin moiety of the acceptor sequence (N-terminal to 14-3-3). B, Top panel. Three different types of 14-3-3 binding sites chosen for detailed study: a conventional basic xxSxP site corresponding to IRS1 S641; an atypical site lacking a basic residue N-terminal to the phospho-threonine corresponding to IRSp53 T340; and a site lacking P(1) but containing D(2) corresponding to GIT2 S415. The Gly-Ala linker sequence is represented in small case as “gba.” All three peptides bind to 14-3-3 only in their phosphorylated but not the nonphosphorylated form (n = 2).

Bottom. Alignment of the 14-3-3 binding sites within human IRSp53. The conserved sites around phospho-T340 and T360 (as shown) allow 14-3-3 binding to block binding of both Cdc42 and SH3 domains to IRSp53 (23). Shown is the sequence alignment of the 14-3-3 binding region for IRSp53 from human, mouse, frog, and zebrafish. Identical residues are indicated by * in the lower row and the corresponding phosphothreonines are in bold. C, An assessment of the peptide overlay efficacy using known 14-3-3 binding sequences (original references are listed in supplementary Table S1A). Shown are typical results of overlays using 14-3-3 and the P(1) and P(2) positions in bold italics. Black bars, 14-3-3 isoform; white bars, 14-3-3 isoform.

Fig. 2. Substitution analysis by 14-3-3 binding to peptide arrays. A, Typical signals generated by peptide arrays based on the IRS-1 pS641 motif with single residue substitutions made at the indicated positions; shown are results from 14-3-3(4) (n = 2). Methionine and cysteine were not included but assumed to be equivalent for binding to leucine and serine respectively. The two other sequences not conforming to the standard motif were also tested (supplementary Fig. S2). For evaluation of synthesis efficiency, the parental sequences were tested in triplicate on each array and standard errors are indicated above the bar corresponding to the parental sequence residue. B, 14-3-3 selectivities for the P(1) and P(2) positions of the three sets of arrays were grouped for comparison. Parental sequences of the library sets are shown along with the P(1) and P(2) positions in bold italics. Black bars, 14-3-3 isoform; white bars, 14-3-3 isoform.
Fig. 3. Screening of potential PKC targets that bind 14-3-3. A, In vivo derived sequences that were predicted and bearing the S/T-x-R motif were synthesized and tested by 14-3-3 overlay (n = 2). Listed are names and sequences of positive binders, with reported phosphorylation sites (*) in the PhosphoSitePlus and PhosphoELM databases (50). Spots 1–4 (in dashed box) are unrelated control sequences. Not shown are sequences with null binding or containing unrelated motifs; the comprehensive array list is detailed in supplementary Table S4. Previously reported interactors are indicated by superscript letters corresponding to the references A (14); B (51). B, Full-length wild-type and mutant Flag-Numbs were expressed in Cos7 cells, immunoprecipitated and tested for the presence of endogenous 14-3-3. The three potential 14-3-3 binding sites (S7, S276, and S295) were predicted by our matrix S/T-x-R (supplementary Fig. S4C) and are known aPKC target sites (27). Protein phosphorylation was maintained by cell treatment with calyculin A prior to cell lysis (10 min). The Scansite values and results of in situ binding are summarized. Shown is representative data of three independent experiments. C, Assessment of cellular 14-3-3 binding proteins that contain PKC-like sites. Streptavidin binding peptide tagged (SBP) 14-3-3 complexes were recovered from transfected Cos7 cells with or without treatment with bryostatin-1 and calcium ionophore A23187. The Western blot using anti-phosphoPKC substrate motif antibody detects several associated proteins bearing the motif enriched in the 14-3-3 complex. Tagged and endogenous (endog.) 14-3-3 proteins are indicated; the data is representative of three independent experiments.
In the test set we found that 14-3-3 bound detectably to 38 of 40 sequences. We did not observe significant differences in specificity among the seven human isoforms toward the test set and therefore chose the 14-3-3/H9256 isoform as representative because it is well analyzed in the literature for binding parameters, and the 14-3-3/H9257 isoform for comparison as it is relatively divergent in primary sequence (Fig. 2C).

The signals from bound 14-3-3 were assigned as weak (+), intermediate (++), and strong (+++). The strongest signals were seen with PCTAIRE-1/2 pS125/pS146 and AANAT pT31, which conform to the canonical binding motif RxxpS/pTxP (15). However, often sequences conform to the canonical motif only at the N-terminal or C-terminal side of the phosphorylated residue (cf. noncanonical highlighted in red) suggesting considerable plasticity in the ligand binding site of 14-3-3. Scansite analyses (14-3-3 mode 1) nonetheless did score the strongest binders of the nonconformers KLC2 pS575 and KLC3 pS465 (supplementary Table S1B) but could not identify IRSp53 pT360. This and IRSp53 pT340 bound 14-3-3 as well as the classical RAF1 pS259 (Fig. 2C). We noted that cysteine-containing peptides (CDC25C pS309 and RIN1 pS351) displayed low reactivity probably because thiol containing residues are synthetically problematic. Thus sequences containing cysteine or methionine were subsequently avoided, usually with serine or leucine substituted respectively.

**Context-dependent Amino Acid Preferences Around the Phosphorylated Residue**—To assess 14-3-3 recognition for different classes of binding sequences, we generated arrays in which amino acid substitutions were made three to four positions N-terminal or C-terminal to the phospho-residue. We chose three different types of 14-3-3 binding sites. Insulin receptor substrate-1 (IRS-1) pS641 (Fig. 2B) exhibits moderate in situ binding and is "canonical" (SPKSvpSAPQII). The other two chosen sequences IRSp53 pT340 and GPCR kinase interacting protein (GIT2) pSer415 are "noncanonical" (supplementary Fig. S2). Proximal to IRS-1 pS641, the position P_1 is clearly more selective than P-1. Proline at P-1 is incompatible with binding (and most disfavored at P-2 and P-3 also). The preference at P_1 for Leu has been noted previously (15), but we observed that P_1 Leu (Met), Phe and Ala are exclusively favored in this context (other residues at P_1 are disfavored). Indeed this position exerts as much influence as P_2 where we found that Pro, Arg, and Trp were strongly preferred. At the P_3 and P_4 positions we find essentially no amino acid preference (data not shown).

For IRSp53 pThr340 (DNYSNpTLPVRS), there are no strong amino acid preferences at P-1, P-2 and P_3 (supplementary Fig. S2) suggesting that 14-3-3 interaction occurs primarily C-terminal to pThr. Comparison of these two sets is informative (Fig. 1B); in the IRS-1 pS641 context Leu, Phe and Ala are exclusively favored in this context (other residues at P_1 are disfavored). Indeed this position exerts as much influence as P_2 where we found that Pro, Arg, and Trp were strongly preferred. At the P_3 and P_4 positions we find essentially no amino acid preference (data not shown).
The sequence surrounding GIT2 pS415 (NNRAKpS1LDSDL) contains the favored Leu at P$_{-1}$ but unusually an acidic amino acid at P$_{-2}$. Again aromatic residues Trp and Tyr were well tolerated at P$_{-1}$, as observed with degenerate peptide libraries (15). The N terminus of this sequence also is more conventional (cf. Arg at P$_{-3}$). Furthermore, the preference of Pro at P$_{-2}$ was far less stringent than the IRS-1 set, with Arg, Phe, and Gly at this position yielding similar signals to the IRSp53 Thr340-derived peptides. For IRSp53 Thr340 we note the P$_{-1}$ binding preference Phe/Ala > Leu/Val > Thr/Ser and for P$_{-2}$ Pro > Phe > Arg/Lys (Fig. 1B). These findings support the notion of peptide conformational flexibility in the binding groove (25). Our array data indicate amino acid preferences that agree with earlier studies using soluble peptides (3, 15), but with additional secondary preferences at P$_{-1}$ and P$_{-2}$. Most notably, Arg was selected for at P$_{-2}$ in addition to Pro in all three array sets. This was unexpected considering the paucity of reported [pS/T]xR sites, as only 8 of 201 mammalian sites surveyed contained a basic residue at P$_{-2}$ (7).

Generation of Search Matrices and in situ Binding Validation—We ranked amino acid preferences from P$_{-2}$ to P$_{-3}$ based on the array data. One clear preference not seen previously involves P$_{-2}$ Arg, which conforms to the consensus motif [S/T]x[R] of many protein kinase C (PKC) substrates, as mentioned later. These rankings (both positive and negative) were used to generate matrices in Scansite format where sequences are fixed around P$_0 =$ Ser/Thr (supplementary Fig. S4). Two versions also fixed P$_{-2}$ as L/M, or the P$_{-2}$ as P/R based on the strong preferences for these residues in our analysis. In our matrices unfavorable residues (inducing loss of binding) were assigned values below 1.0, and positive residues scored 1–20 (the algorithm applies a natural log to calculate overall score). We assigned the rarer amino acid Met and Cys (which were not directly assessed) with values equal to Leu or Ser, respectively.

To assess prediction efficacy, we selected and tested 272 phospho-sequences predicted by our matrices (supplementary Table S2). Overall, 80% were positive for 14-3-3 in situ binding. We also considered known in vivo phosphorylated sites (supplementary Table S3) curated in the PhosphoSitePlus and PhosphoELM databases. Of 80 such sequences primarily with L(+1) or P(+2) sequences but with widely varying Scansite scores, 38 were positive for 14-3-3 binding (45%; supplementary Fig. S3). Of these positives, 13 were reported as 14-3-3 interacting proteins (supplementary Table S3), but only two sites have been mapped (PCTAIRE1 Ser125; AKT1S1 Ser183).

A Widespread Association of 14-3-3 with [pS/T]xR Containing Proteins—To confirm 14-3-3 in situ binding with a wide variety of sequences bearing [S/T]x[R] we tested 34 human sequences predicted by our S/TxR matrix, with three [S/T]x[P] bearing sequences included for comparison (Fig. 3A). PAPOLB S223 (spots 17, 18) and CK1δ S257 sequences (spots 31, 35) were tested in duplicate for synthesis controls. The M371L substituted CK1δ S370 sequence exhibited better binding than the parental sequence (spots #54, 55) suggesting higher peptide yield. All sequences interacted with 14-3-3, but only two of these proteins are reported as 14-3-3 interacting proteins (PAK4 and APC). Human Numb has been identified as a 14-3-3 binding protein (26) and is regulated by PKC, but no other PKC-like sites other than its homolog NumbI are identified in the recent analysis of 201 mammalian 14-3-3 binding sites (7). Using the S/TxR matrix, we scored Numb Ser295, Ser276, and Ser7 as the top three predicted 14-3-3 binding sites. These sites are targeted by aPKC (27). Wild-type Numb and Numb(S7A) associated with endogenous 14-3-3 under basal conditions with significantly increased levels after calyculin A stimulation, but Numb(S276A) and Numb(S295A) barely showed any association in treated or untreated cells (Fig. 3B).

To address a more global association with phospho-[S/T]xR sequences in proteins, 14-3-3$_3$ tagged with streptavidin binding peptide (SBP) was transiently expressed in Cos7 cells and coprecipitated proteins were examined by Western blotting using an “anti-phospho-PKC substrate” [Rxx[S/T]xP/R/K] antibody (Fig. 3C). Even in unstimulated cells, many immuno-positive proteins bound SBP-14-3-3$_3$, but there was a clear increase in level and numbers of associated bands after treatment with the PKC agonist bryostatin-1 and A23187 (calcium ionophore). We conclude that certainly a subset of the PKC-like site at S657 is therefore one of several 14-3-3 binding sites; of the known phosphorylation sites on MARK3 (29), 5/7 were positive for 14-3-3 binding by peptide overlay. A study has shown singly substituted Ser/Thr to Ala MARK3 mutants behave as wild type, and only when all 17 candidate Ser/Thr mutations was 14-3-3 binding abolished (29). The PKC-like site at S657 is therefore one of several 14-3-3 binding sites.

Measuring 14-3-3 Binding Affinity for Noncanonical Phospho-peptides—To confirm that the results obtained by overlay translate to affinity binding constants for 14-3-3 in the range previously described, we assessed equilibrium binding using SPR with three peptides that do not have canonical Rxx[S/T]xP/R/K sequences and yield “moderate” binding signals by overlay. The sequences corresponded to: GIT2 pS415, PAR6 pS44, and GCK pS170 (Fig. 4). Peptides were biotinylated using a carboxyl lysine (side chain) and immobilized using streptavidin tetramer. Purified His$_6$-14-3-3$_3$ bound all three
peptides with submicromolar affinities, with the PAR6 showing highest affinity ($K_d = 0.33 \mu M$). These values are comparable with published affinity constants for well characterized peptides such as the Raf-1 pS259 motif ($K_d = 0.12 \mu M$) (2, 15) and the p53 pS366/p378 sequence ($K_d = 0.48 \mu M$) (30). From the peptide array analysis (Fig. 1, supplementary Fig. S2) we suggest the dominant feature is Leu$^1$, with hydrophobic or basic residues contributing at positions -1, -2, and -3, particularly in the absence of Pro$^2$. We note that the association phases were used for equilibrium binding analysis were relatively slow ($t_{1/2}$ range 2–8 min; Fig. 4) perhaps reflecting conformational reorganization needed to adopt two-site binding. This stability of the 14-3-3:phospho-peptide complex was evident in the slow dissociation rates. A slow exchange rate between 14-3-3 and target phospho-peptide has been reported (31). Further structural analysis using phospho-proteins, which are more conformationally constrained than peptides, is clearly required.

A Re-evaluation of 14-3-3 Binding to Cdc25C—To evaluate the efficacy of a peptide-based protocol to reveal 14-3-3 binding sites, we re-evaluated the well established interaction between 14-3-3 and Cdc25C. The phospho-dependent binding of 14-3-3 is central to cytoplasmic-nuclear localization of Cdc25C during the cell cycle, which has been extensively studied in Xenopus oocytes (32–35) and mammalian cells (36–39). To date only one 14-3-3 binding site has been identified in Xenopus (34, 35) and mammalian Cdc25C (36, 38). We used in silico prediction to identify other potential sites which are conserved across species (Fig. 5); Ser247 is not predicted to bind 14-3-3 but was considered here because it lies within a region suggested as the nuclear localization sequence (40). The results of the overlay assay are summarized in Fig. 5A. We found that Cdc25C(S263A) and (S247A) mutants were much reduced in association with endogenous 14-3-3 (bottom panel). Importantly, these mutants were not altered in their modification at Ser216 site in asynchronous cells (Fig. 5B) but both were shifted downwards (by SDS-PAGE) suggesting both sites are significantly modified in the cell. Mass spectrometry has shown that Cdc25C Ser263 is phosphorylated in vivo (41). It has been clearly shown that phosphorylation of this residue is needed for cytoplasmic retention of Cdc25C (in addition to Ser216); the S263A substitution thus leads to the protein shifting to the nucleus (41), although the basis for this effect was never established.

Fig. 5. Identification of new sites required for 14-3-3 binding to Cdc25C. A, Top panel: schematic diagram of the domains and motifs found in human Cdc25C including the known site Ser216. Additional potential sites were scored using matrices S/T-L/M and S/T-x-P (supplementary Fig. S4), or Scansite 14-3-3. Top scoring results are listed and those tested by in situ binding are indicated. Sites marked by * are the Cdc25B homologs to the Cdc25C sequence directly above. Mammalian Cdc25C is widely reported as a 14-3-3 binding protein. Middle panel: Alignment of the identified 14-3-3 binding sites and overlapping NLS regions in human and Xenopus Cdc25C, compared with the same region in human Cdc25B. Identical residues are indicated in bold, similar residues in bold gray and known phosphorylated residues in red. The reported nuclear localization sequence (40) and start of phosphatase domain are indicated. Bottom panel: full-length wild-type Cdc25C or the S247A and S263A mutants were immunoprecipitated from unsynchronized cells and tested for endogenous 14-3-3 proteins. Western blots were performed on control or calyculin A treated cells. These results are representative of three independent experiments. B, Modification of Cdc25C pS216 site is unaffected by mutation of S247A and S263A. Cell lysates as in (A) were probed by anti-pS216 Cdc25C antibody ($n = 3$).
Alignment of human and frog Cdc25C proteins showed 91% similarity in the S216 motif, 45% in the S247 motif and 91% in the S263 motif (Fig. 5A, yellow boxed regions). These motifs are conserved in human Cdc25B, and the S263 site is conserved in the Drosophila Cdc25 ortholog string (data not shown), pointing to a biological function.

DISCUSSION

Based on an assessment of the number of nonoverlapping target sites reported from recent proteomic studies (8), an estimate of ~1–2% of proteins can potentially bind human 14-3-3 in varying cellular conditions. Database searching using Scansite yields 14-3-3 target sequences with high probability scores in ~2% of all human sequences, but the number of proteins with two of such sites is much smaller. Because serine and threonine residues are highly represented in the phospho-proteome (3), and 14-3-3s are relatively promiscuous toward target phosphosites (7, 8, 42), identification of binding sites can be time consuming and incomplete. Further, the distance between tandem sites that bind a 14-3-3 dimer cannot be predicted from primary sequence. Thus, accurate binding site prediction remains a bottleneck, with the majority of known 14-3-3 target proteins yet to be mapped. Given that 14-3-3 binding plays a key regulatory role in protein function, site identification is a functionally important goal. We describe here a protocol to help accelerate this discovery.

The higher sensitivity of our array format to single amino acid changes versus chemical sequencing of selected pooled peptides (18), likely results from bidentate nature of the binding of 14-3-3 to the arrays. It has been demonstrated with a gad SH3 domain probe that signals generated in overlays with peptides bearing single amino acid substitutions in synthetic arrays correlate well with measured binding affinities (43). Our arrays revealed 14-3-3 secondary preferences, particularly Arg at the P1 position (Fig. 1; supplementary Fig. S2). We searched the SwissProt human database and found 4176 sequences for the canonical motif Rxxx[PS/T]xP versus 3916 for the alternate motif Rxxx[PS/T]xR, showing that both are comparably represented. Earlier studies of 14-3-3 recognition have generated user-friendly prediction tools (16) but over time we do not know the extent of bias introduced into mapping studies, particularly if sites are chosen for testing primarily based on historical data. For example, a recent survey of ~200 reported binding sites showed that approximately half contained the conventional Pro1 motif and only three sites from two nonhomologous proteins contained the alternate Arg1 motif (7). Whether this disproportionately represents a true profile of in vivo sites is largely unclear, because we suspect there is overselection of the Pro1 motif among the candidate sites. Assessment of the set of known binding sites (Fig. 2) shows that some are missed by the existing Scansite analysis and binding levels have no clear correlation with scores (supplementary Table S1B). We confirmed 14-3-3 in situ binding to 228 sequences that are currently unreported to our knowledge (supplementary Tables S2) and in which a significant proportion does not contain Pro1. We also found that non-Pro1-containing peptides had submicromolar affinities comparable to reported canonical sequences (Fig. 4). These data support the notion of higher coverage for 14-3-3 than currently appreciated.

Using the matrix based on an alternate motif (supplementary Fig. S4C), we confirmed binding sites on Numb (Fig. 3), a target of aPKC (27). Either of the top two scoring sites S295 and S276 was required for 14-3-3 association, whereas the third-ranked site S7 may not mediate binding or was not phosphorylated under the conditions. Numb localization is excluded from the aPKC complex in Drosophila sensory organ precursor cells (44) and in dividing neuroblasts (45). Similarly, aPKC activity and 14-3-3 interaction drives phospho-MARK2 translocation away from the basolateral membrane resulting in the mutual exclusion of the aPKC complex and MARK2 in epithelial cells (46). For proteins with numerous 14-3-3 binding sites such as MARK2, the peptide array/overlay method can be used to parse out the contributing sites (supplementary Fig. S5) whereas mutational analysis of the complete protein is much more complex and uninformative (29). We also showed that in silico prediction can aid in validating 14-3-3 targets containing at least two binding sequences (supplementary Fig. S5), namely PCTAIRE1, AKT1S1, and PRKCDDBP, with the latter not predicted by the existing Scansite analysis (more details in supplemental text).

The results with Cdc25C, which has long been assumed to contain a single binding site, raise the question as to how many sites have been missed in “well characterized” 14-3-3 regulated proteins. Numerous studies have confirmed the phosphorylation of Ser216 of Cdc25C as a critical requirement for 14-3-3 binding (36–38, 40, 47). As pSer216 status was unperturbed in the Cdc25C mutants (Fig. 5B) and 14-3-3 site function as dimers (19) we conclude that pSer263, which has been reported to be phosphorylated in vivo (41), plays a key role (with pSer216) in promoting 14-3-3 association with Cdc25C. Phosphorylation at Ser263 has been shown to regulate Cdc25C nuclearcytoplasmic shuttling (41) as a Ser to Ala substitution shifted the mutant protein to the nucleus in 70% of asynchronously dividing cells for wild-type Cdc25C; the same study also reported the homologous Ser375 of Cdc25B to be phosphorylated in dividing cells. The S247 site, which has not been reported in the phosphorylation site databases, lies between these two phospho-sites and may play a conformational role in modulating 14-3-3 binding; alternatively it may function as a priming site for efficient phosphorylation of Ser263. Whereas 14-3-3 binding has been shown to be absolutely required for efficient Cdc25C sequestration from its nuclear substrates during interphase and prevention of premature mitosis (38), the level of 14-3-3 binding...
apparently does not regulate the phosphatase activity of Cdc25C toward its cellular substrates (48).

In summary, we showed that our approach combining enhanced bioinformatics prediction tools and in situ binding can rapidly reveal 14-3-3 binding sites that are not predicted by current methods, as observed for Cdc25C. This would greatly aid in validating target sites within full-length proteins of the steadily expanding 14-3-3 interactome, of which the vast majority is still singly mapped or unmapped. We observed no significant differences in specificity among the seven human isoforms toward the test set (Fig. 1) nor among four isoforms toward the known phosphosite set (supplementary Table S3). This is consistent with previous studies of 14-3-3 isoforms using single target peptide sequences (2) or pooled peptides (15), which showed highly conserved binding parameters and sequence preferences, respectively. Therefore under in vitro conditions, 14-3-3 isoforms exhibit similar binding characteristics toward synthetic peptides. As our prediction matrices (supplementary Fig. S4) are derived from such in vitro binding data, their usage would not be isoform restricted. The simple work-flow for site mapping described here can be combined with peptide accessibility prediction methods (49) to improve detection of candidate sites.

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REFERENCES

1. Moore, B. W., and Perez, V. J. (1967) Specific Acid Proteins in the Nervous System. Physiological and Biochemical Aspects of Nervous Integration. 343-359, Prentice Hall, New Jersey, U.S.A.

2. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell 84, 889–897

3. Hunter, T., and Setton, B. M. (1988) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77, 1311–1315

4. Morrison, D. K. (2009) The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. Trends Cell Biol. 19, 16–23

5. Dougherty, M. K., and Morrison, D. K. (2004) Unlocking the code of 14-3-3. J. Cell Sci. 117, 1875–1884

6. Gardino, A. K., Smerdon, S. J., and Yaffe, M. B. (2006) Structural determinants of 14-3-3-binding specificities and regulation of subcellular localization of 14-3-3-ligand complexes: a comparison of the X-ray crystal structures of all human 14-3-3 isoforms. Semin. Cancer Biol. 16, 173–182

7. Johnson, C., Crowther, S., Stafford, M. J., Campbell, D. G., Toth, R., and MacIntosh, C. (2010) Bioinformatic and experimental survey of 14-3-3-binding sites. Biochem. J. 427, 69–78

8. Bridges, D., and Moorhead, G. B. (2005) 14-3-3 proteins: a network of functions for a numbered protein. Sci. STKE 2005, re10

9. Aitken, A., Collinge, D. B., van Heusden, B. P., Isobe, T., Roseboom, P. H., Rosenfeld, G., and Soll, J. (1992) 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. Trends Biochem. Sci. 17, 498–501

10. Jin, J., Smith, F. D., Stark, C., Wells, C. D., Fawcett, J. P., Kulkarni, S., Metalnikov, P., O’Donnell, P., Taylor, P., Taylor, L., Zouman, A., Woodgett, J. R., Langeberg, L. K., Scott, J. D., and Pawson, T. (2004) Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytokesin regulation and cellular organization. Curr. Biol. 14, 1436–1450

11. Pozuelo Rubio, M., Geraghty, K. M., Wong, B. H., Wood, N. T., Campbell, D. G., Morrice, N., and Mackintosh, C. (2004) 14-3-3 affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. Biochem. J. 379, 395–408

12. Manser, E., Lane, W. S., and Pwicna-Worms, H. (2004) Comprehensive proteomic analysis of interphase and mitotic 14-3-3-binding proteins. J. Biol. Chem. 279, 32046–32054

13. Angrand, P. O., Segura, I., Vogel, P., Ghielmetti, S., Terry, R., Brajevoc, M., Vintersten, K., Klein, R., Superti-Furga, G., Drewes, G., Kuster, B., Bouwmeester, T., and Acker-Palmer, A. (2006) Transgenic mouse proteomics identifies new 14-3-3-associated proteins involved in cytokesin signaling. Mol Cell Proteomics 5, 2211–2227

14. Benzinger, A., Muster, N., Koch, H. B., Yates, J. R., 3rd, and Hemarksen, H. (2005) Targeted proteomic analysis of 14-3-3 sigma, a ps3 effector commonly silenced in cancer. Mol Cell Proteomics. 4, 785–795

15. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) The structural basis for 14-3-3phosphopeptide binding specificity. Cell 91, 961–971

16. Oberha user, J. C., Cantley, L. C., and Yaffe, M. B. (2003) Scansite 2.0—Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. 31, 3635–3641

17. Brune, D. C., Hampton, B., Kobayashi, R., Le neuron, L. W., Linse, K. D., Pohl, J., Thoma, R. S., and Denslow, N. D. (2007) ABFR ESRG 2006 study: Edman sequencing as a method for polypeptide quantitation. J Biomol Tech 18, 306–320

18. Thoma, R. S., Smith, J. S., Sandoval, W., Le neuron, J. W., Hunsiker, P., Hampton, B., Linse, K. D., and Denslow, N. D. (2009) The ABFR Edman Sequencing Research Group 2008 Study: investigation into homopolymeric amino acid N-terminal sequence tags and their effects on automated Edman degradation. J Biomol Tech 20, 216–225

19. Fu, H., Subramanian, R. R., and Masters, S. C. (2000) 14-3-3 proteins: structure, function, and regulation. Annu. Rev. Pharmacol. Toxicol. 40, 17–47

20. Yeow-Fong, L., Lim, L., and Manser, E. (2005) SNX9 as an adaptor for linking pantothenoate-1 to the Cdc42 effector ACK1. FEBS Lett. 579, 5040–5048

21. Yaffe, M. B., Leparc, G. G., Lai, J., Obata, T., Volinia, S., and Cantley, L. C. (2001) A motif-based profile scanning approach for genome-wide prediction of signaling pathways. Nat Biotechnol 19, 348–353

22. Wilson, D. S., Keefe, A. D., and Szo staw, J. W. (2001) The use of mRNA display to select high-affinity protein-binding peptides. Proc. Natl. Acad. Sci. U.S.A. 98, 3750–3755

23. Robens, J. M., Lee, Y. F., Ng, E., Hall, C., and Manser, E. (2010) Regulation of IRSp53-dependent filopodial dynamics by antagonism between 14-3-3 binding and SH3-mediated localization. Mol. Cell. Biol. 30, 829–844

24. Yang, X., Lee, W. H., Sobott, F., Papagrigoriou, E., Robinson, C. V., Grossmann, J. G., Sundstrom, M., Doyle, D. A., and Elkins, J. M. (2006) Structural basis for protein-protein interactions in the 14-3-3 protein family. Proc. Natl. Acad. Sci. U.S.A. 103, 17237–17242

25. Ohsib, T., Ghi lamando, R., Klein, D. C., Ganguly, S., and Dyda, F. (2001) Crystal structure of the 14-3-3zetaratutamin N-acetyltransferase complex. a role for scissioning in enzyme regulation. Cell 105, 257–267

26. Tokumitsu, H., Hatano, N., Huzuka, H., Sueyoshi, Y., Yokokura, S., Ichimura, T., Nozaki, N., and Kobayashi, R. (2005) Phosphorylation of Numb family proteins. Possible involvement of Ca2+/calmodulin-dependent protein kinases. J. Biol. Chem. 280, 35108–35118

27. Smith, C. A., Lau, K. M., Rahmani, Z., Dho, S. E., Brothers, G., She, Y. M., Berry, D. M., Bonneil, E., Thibault, P., Schwesiguth, F., Le Borgne, R., and McGlade, C. J. (2007) aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. EMBO J. 26, 468–480
28. White, R. R., Kwon, Y. G., Taing, M., Lawrence, D. S., and Edelman, A. M. (1998) Definition of optimal substrate recognition motifs of Ca2+–calmodulin-dependent protein kinases IV and II reveals shared and distinctive features. J. Biol. Chem. 273, 3166–3172

29. Göransson, O., Deak, M., Wullschleger, S., Morrice, N. A., Prescott, A. R., and Alesil, D. R. (2006) Regulation of the polarity kinases PAR-1/MARK by 14-3-3 interaction and phosphorylation. J. Cell Sci. 119, 4059–4070

30. Rajagopalan, S., Jaulent, A. M., Wells, M., Veprintsev, D. B., and Fersht, A. R. (2008) 14-3-3 activation of DNA binding of p53 by enhancing its association into tetramers. Nucleic Acids Res. 36, 5983–5991

31. Silhan, J., Obsilova, V., Vecer, J., Herman, P., Sulc, M., Teisinger, J., and O&Osl, T. (2004) 14-3-3 protein C-terminal stretch occupies ligand binding groove and is displaced by phosphopeptide binding. J. Biol. Chem. 279, 49113–49119

32. Yang, J., Winkler, K., Yoshida, M., and Kornbluth, S. (1999) Maintenance of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76, 477–491

33. Mackintosh, C. (2004) Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. Biochem. J. 381, 329–342

34. Suzuki, A., Hirata, M., Kamimura, K., Maniwa, R., Yamanaka, T., Mizuno, K., Kishikawa, M., Hirose, H., Amano, Y., Izumi, N., Miwa, Y., and Ohno, S. (2004) APKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. Curr. Biol. 14, 1425–1435

35. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997) Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 277, 1497–1501

36. Blasina, A., de Weyer, I. V., Laus, M. C., Luyten, W. H., Parker, A. E., and McGowan, C. H. (1999) A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr. Biol. 9, 1–10

37. Wagner, M., Adamczak, R., Porollo, A., and Meller, J. (2005) Linear regression models for solvent accessibility prediction in proteins. J Comput Biol 12, 355–369

38. Biolo, F., Gould, C. M., Chica, C., Via, A., and Gibson, J. T. (2008) Phospho.ELM: a database of phosphorylation sites—update 2008. Nucleic Acids Res. 36, D240–244

39. Ewing, R. M., Chu, P., Elisma, F., Li, H., Taylor, P., Clime, S., McBroome-Cerajewski, L., Robinson, M. D., O’Connor, L., Li, M., Taylor, R., Dharsee, M., Ho, Y., Heilbut, A., Moore, L., Zhang, S., Ormatsky, O., Buhkman, Y. V., Ethier, M., Sheng, Y., Vasilescu, J., Abu-Farha, M., Lambert, J. P., Duewel, H. S., Stewart, I. I., Kuehl, B., Hogue, K., Colwill, K., Gladwisch, K., Muskat, B., Kinach, R., Adams, S. L., Morin, M. F., Morin, G. B., Topaloglou, T., and Figeys, D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol Syst Biol 3, 89

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