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Systematic investigation of hierarchical phosphorylation by protein kinase CK2☆

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ABSTRACT

Although multiple phosphorylation sites are often clustered in substrates, the mechanism of phosphorylation within clusters has not been systematically investigated. Intriguingly, in addition to acidic residues, protein kinase CK2 can use phosphoserine residues as consensus determinants suggesting that CK2 may act in concert with other kinases. We used a peptide array approach to outline optimal consensus sequences for hierarchical phosphorylation by CK2, both in the context of processive, multisite phosphorylation, and in concert with a priming proline-directed kinase. Results suggest that hierarchical phosphorylation involving CK2 requires precise positioning of either multiple phosphodeterminant residues or specific combinations of canonical determinants and phosphodeterminants, and can be as enzymatically favorable as canonical CK2 phosphorylation. Over 1600 human proteins contain at least one CK2 hierarchical consensus motif, and ~20% of these motifs contain at least one reported *in vivo* phosphorylation site. These motifs occur non-randomly in the human proteome, with significant enrichment in proteins controlling specific cellular processes. Taken together, our results provide strong *in vitro* evidence that hierarchical phosphorylation may contribute to the regulation of crucial biological processes. In addition, the results suggest a mechanism by which CK2, a constitutively active kinase, can be a regulatory participant in cellular processes.

Biological significance
Phosphorylation is a crucial regulatory mechanism governing cellular signal transduction pathways, and despite the large number of identified sites to date, most mechanistic studies remain focused on individual phosphorylation sites. This study is the first to systematically determine specific consensus sequences for hierarchical phosphorylation events. The results indicate that individual phosphorylation sites should not be studied in isolation, and that larger, multisite phosphorylation motifs may have profound impact on cellular signaling. This article is

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1. Introduction

By 1979, when Beavo and Krebs published a set of guidelines for the establishment of kinase–substrate interactions, just over twenty proteins had been identified as regulated through reversible phosphorylation [1]. With no way of knowing how widespread, and crucial to cellular signaling, phosphorylation would prove to be, the authors optimistically speculated that "… if the present trend continues, another 10–15 [substrates] could be added to the group within the next five years." [1]. Several decades of work later, we possess a huge body of knowledge regarding kinase specificity, kinase–substrate reactions, and their individual functional effects on the cell. It is estimated that one third of intracellular proteins are phosphorylated, many on several distinct sites [2], and large scale phosphoproteomic screens have contributed large datasets of potential phosphorylation sites (for example, as of June 2014, the PhosphoSitePlus database [3] lists almost 120,000 human phosphorylation sites). Interestingly, global analysis of known phosphorylation sites in humans and mice demonstrates significant clustering of phosphorylation sites into specific regions, often showing concurrent phosphorylation [4]. Strikingly, in a study of 70,000 *in vivo* phosphorylation sites, 54% of phosphoserine (pS) and phosphothreonine (pT) residues were located no further than four amino acids from another pS/pT residue [5]. While it is apparent that the regulated phosphorylation of proteins is intricately involved in every fundamental cellular process [6], our understanding of the interplaybetween distinct phosphorylation sites has lagged considerably.

Hierarchical protein phosphorylation is a phenomenon in which a kinase phosphorylates a substrate based on its unique sequence determinants, and the addition of phosphate creates adequate sequence determinants for nearby phosphorylation events to occur. These events can result in processive phosphorylation events catalyzed by one kinase, or can involve two or more distinct kinases. Protein kinase families such as GSK3 or CK1 catalyze primed phosphorylation events almost exclusively, as phosphorylation by these kinases usually requires prior phosphorylation of a nearby residue [7,8]. By comparison, protein kinase CK2 seems to be more distinct in its ability to use either non-phosphorylated or phosphorylated determinants for phosphorylation [9]. Interestingly, CK2 may therefore be capable of generating clusters of phosphorylation sites both independently and in concert with other kinases. CK2 is a ubiquitously expressed serine/threonine kinase with a multitude of substrates. It participates in a variety of cellular processes, including proliferation, apoptosis, transcription, and translation [10]. In fact, CK2 phosphorylation is so widespread that an estimated 20% of the phosphoproteome can be attributed to CK2 on the basis of phosphopeptides that conform to the minimal consensus recognition sequence for CK2 [11]. CK2 is an acidophilic kinase, with canonical (non-hierarchical) CK2 phosphorylation requiring one or more acidic residues C-terminal to the phosphoacceptor site. Accordingly, the minimal consensus sequence for CK2 phosphorylation is S/T–X–X–D/E. While X can be any amino acid, studies have shown that proline, lysine, or arginine at the +1 position are unfavorable [12,13]. In some instances, phosphorylation by CK2 is enabled by an acidic determinant in the +1 position instead of the +3 position. Multiple aspartic or glutamic acid residues seem to have an additive effect, and due to this, many known CK2 sites consist of a serine/threonine residue followed by a string of acidic residues [14]. It has long been recognized that phosphoserine (pS) can substitute for the acidic determinant at the +3 position, enabling CK2 to participate in hierarchical signaling events [9,15,16]. However, the precise consensus requirements for these events, as well as their impact on cellular signaling, have not been thoroughly investigated. In this study, we use a peptide-based approach to outline favorable consensus requirements for hierarchical phosphorylation by CK2. The results indicate that efficient hierarchical phosphorylation by CK2 requires either multiple phosphoserine residues, or a mix of canonical and hierarchical determinants, with precise spacing. Kinetic analysis indicates that these reactions may be as enzymatically favorable as canonical phosphorylation. Using these determinants, a search of the human proteome for CK2 hierarchical consensus sequences revealed over 1600 proteins that contain at least one motif for CK2 hierarchical phosphorylation, with significant enrichment for proteins involved in transcriptional regulation, development, differentiation, and other fundamental processes. Notably, a number of these sites are previously reported *in vivo* phosphorylation sites. These results provide compelling *in vitro* evidence that hierarchical phosphorylation by CK2 has the potential to regulate several crucial cellular processes, demonstrating the impact that hierarchical phosphorylation could have on signal transduction.

2. Materials and methods

2.1. Purification of active CK2 holoenzyme

CK2 holoenzyme, consisting of GST-CK2α and His-CK2β, was purified from bacterial culture as previously described [17]. Enzyme concentration was determined by absorbance at 595 nm, measured on a Victor3 V 1420 multilabel counter (Perkin Elmer) using BSA standards. The enzyme was diluted (up to 1:5000) in CK2 Dilution Buffer (5 μM MOPS pH 7.0, 200 mM NaCl, 1 mg/mL BSA) immediately before use in kinase assays.

2.2. Peptide kinase assays

Peptides were synthesized using standard Fmoc (9-fluorenyl methyloxycarbonyl) chemistry at a 2 μmol scale on an Intavis Multipep Synthesizer. The sequences of all peptides used in

this study can be found in Supplemental Table S1. To enable determination of peptide concentration by absorbance at 280 nm, peptides were synthesized with an N-terminal tryptophan residue. Biotin was added to the N-terminus of each peptide to enable capture by streptavidin. Peptides used in the multisite walking array (Fig. 3) contained three N-terminal arginine residues instead of biotin, for capture on P81 paper (Whatman). Peptides were cleaved from the resin using trifluoroacetic acid, and peptide identities were confirmed by mass spectrometry. Peptides were resuspended in DMSO and concentrations were determined by absorbance at 280 nm. Peptide concentration was adjusted to 50 mM, and aqueous 2.5 mM stock solutions were made in 20 mM HEPES pH 7.4. Kinase assays were performed for 10 min at 30 °C in a final reaction volume of 10 μl containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1 mM DTT, 0.1 mM ATP, and 1 μCi [γ^{32}P]-ATP (specific activity 3000 Ci/mol, Perkin-Elmer). Substrate peptides were used at 0.5 mM unless otherwise indicated. Reactions were initiated by the addition of CK2, and stopped by the addition of 0.1 M EDTA (pH 8.0) followed by spotting 4 μl aliquots of each reaction onto either a SAM2 streptavidin membrane (Promega) or P81 paper. The SAM2 membrane was washed twice in 0.1% SDS in TBS, twice in 2 M NaCl, twice in 2 M NaCl and 1% H_3PO_4, and twice in distilled water, and then dried under a heat lamp. The P81 paper was washed 3 times in 1% phosphoric acid, once in ethanol, and then dried under a heat lamp. Following exposure to a phosphor storage screen, CK2 phosphorylation was visualized using a Storm Phosphorimager (Molecular Dynamics) and [γ^{32}P] incorporation for each peptide was determined using ImageQuant TL software (Amersham Biosciences). All phosphorylation assays were performed in triplicate. The z score for each peptide (which represents the number of standard deviations by which the sample peptide phosphate incorporation differs from that of a negative control (SAAAAA)) were calculated using the formula $z=(x-\mu)/\sigma$, where x and μ represent the mean phosphate incorporation for the sample peptide and the control peptide respectively, and σ represents the standard deviation of the negative control peptide. For kinetic studies, each peptide was assayed for phosphorylation at a minimum of six different concentrations, and five replicates of each concentration were used in the analysis. Km and Vmax values for each peptide were determined by nonlinear regression analysis fit to a Michaelis–Menten model for enzyme kinetics using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Each dataset was subjected to a replicate test for lack of fit, and the Michaelis–Menten model was deemed adequate for all peptides studied.

2.3. Peptide dephosphorylation

Biotinylated hierarchical peptides (1 mM) were dephosphorylated with 400 U lambda phosphatase (New England Biolabs) in 50 mM HEPES (pH 7.5), 10 mM NaCl, 2 mM DTT, 1 mM $MnCl_2$, for 1 h at 30 °C, followed by heat inactivation for 1 h at 95 °C. Complete dephosphorylation of peptides was determined by mass spectrometry. Control reactions were performed in the absence of lambda phosphatase. Kinase assays were then performed as above. All dephosphorylation and phosphorylation assays were performed in triplicate.

2.4. Bioinformatics

A peptide match program was designed to search the NCBI non-redundant human proteome (accessed 14/05/15) for human proteins containing potential hierarchical phosphorylation events. The program was engineered to search for peptides matching the multisite phosphorylation sequence [ST]S[ADEG]S[ADEGLIVFHNQ][ADEGLIVFHNQS][ADEGLIVHS] or the proline-directed sequences S[DE]X[DE]XSP and [ST][ACDEFGHILMNQSTVWY]X[DE]XSP, where X is any amino acid. For proteins with a matching motif, the program returned the GI number, protein name, peptide sequence, and position within the protein. The PhosphoSitePlus [3] phosphorylation database (accessed June 10, 2014) was searched to determine if the resultant peptide sequences were previously reported to be phosphorylated, and the results were analyzed for enrichment in Biological Function (GOTERM_BP_ALL), Molecular Function (GOTERM_MF_ALL), and Conserved Domains (INTERPRO) using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [18,19] (accessed 14/06/12). The Benjamini–Hochberg coefficient was used as the false discovery rate (FDR), with a significance cutoff of 0.05. Enrichment Map [20] was used to map the results in Cytoscape [21], with a maximum P value of 0.005, a maximum FDR of 0.1, and minimum similarity score of 0.5. Conserved domain information for selected hits was extracted from the NCBI Conserved Domains Database [22].

3. Results and discussion

3.1. Hierarchical phosphorylation by CK2 requires the precise positioning of multiple phosphoserine residues

The canonical consensus sequence for CK2 phosphorylation is largely dependent on acidic residues at specific positions (typically +1 and/or +3), but the CK2 phosphorylation sites of strongest catalytic efficiency have a continuous string of acidic residues following the phosphoacceptor [14]. This suggests that for canonical phosphorylation sites, extra acidic residues are favorable. To determine if this was true for hierarchical phosphorylation as well, we designed an array of peptides representing all possible combinations of alanine and phosphoserine at the +1 to +5 positions relative to a phosphoacceptor serine (parent peptide: WDDDSpSpSpSpSpSAAA, the phosphoacceptor site is underlined). CK2 phosphorylation of these substrates varied widely, even among peptides with the same total amount of phosphoserine (Fig. 1A). In fact, phosphorylation of the parent peptide (SpSpSpSpSpS) was barely detectable, and removal of almost any single phosphoserine improved phosphorylation. In general, the optimal number of phosphoserines for hierarchical phosphorylation appears to be 2–3, as the only peptide containing just one phosphoserine to be phosphorylated above background was the previously published hierarchical sequence, SAApSAA [9]. However, among peptides with 2–3 phosphoserine residues, phosphorylation of different combinations of phosphoserine and alanine varied appreciably, suggesting that, as in the canonical consensus sequence, the precise positioning of

phosphoserine is important for effective phosphorylation of a hierarchical CK2 consensus sequence.

As is the case for canonical phosphorylation, hierarchical phosphorylation by CK2 seems to be almost completely dependent on phosphodeterminants at the +1 and +3 positions (Fig. 1B). In fact, all significantly phosphorylated peptides ($z \geq 1.95$; corresponding to $P \leq 0.05$) in this study had at least one phosphoserine at either the +1 or +3 position, and of the 11 peptides that were not significantly phosphorylated by CK2, only 4 peptides contained a phosphoserine at either the +1 or +3 position. In addition to the +1 and +3 positions, additional acidity at the +4 or +5 position typically increased CK2 phosphorylation. In contrast, the presence of a phosphoserine residue at the +2 position had at best a negligible effect on CK2 phosphorylation, and in many cases was inhibitory. For example, the SpSpSpSAA peptide displays only weak phosphorylation by CK2, but with an alanine instead of the +2 phosphoserine, the SpSApSAA peptide was one of the strongest peptides in the study. Acidic determinants at the +2 position are typically negligible for canonical signaling as well [23], but inhibitory effects seem to be unique to hierarchical phosphorylation. Three peptides displayed particularly strong phosphorylation by CK2: SpSApSpSA, SpSApSApS, and SpSApSAA. From this, we conclude that the optimal consensus sequence for hierarchical CK2 phosphorylation will have phosphoserines at +1 and +3, with a possible additional phosphoserine at either +4 or +5.

The similarities in spacing requirements are due to the geometry of the CK2 substrate binding cleft, which uses three distinct basic regions of the kinase to coordinate acidic determinants for phosphorylation into the active site, positioning the phosphoacceptor residue for catalysis [23,24]. However, while canonical sites typically contain multiple acidic determinants [14], too many phosphodeterminants inhibit CK2 phosphorylation, with an upper limit of 2–3 sites. This may be explained by the fact that while aspartic acid and glutamic acid, with pKa values of 3.9 and 4.07, respectively, will each have one negative charge at physiological pH, each phosphoryl group, with a pKa of 6.7, will most likely have two negative charges [25]. Since binding of CK2 to its substrates appears to be largely dependent on negative charges in the substrate, we propose that CK2 sites may have an upper threshold of negativity, above which the high number of negative charges may interfere with substrate phosphorylation. This idea is supported by mutational studies performed on the CK2 substrate binding cleft, as mutation of certain basic residues to alanine results in decreased CK2 phosphorylation due to an increase in Km, meaning that CK2 can no longer effectively bind its substrates [23,24]. This may also explain why phosphodeterminants at the +2 position actually decrease primed phosphorylation: the

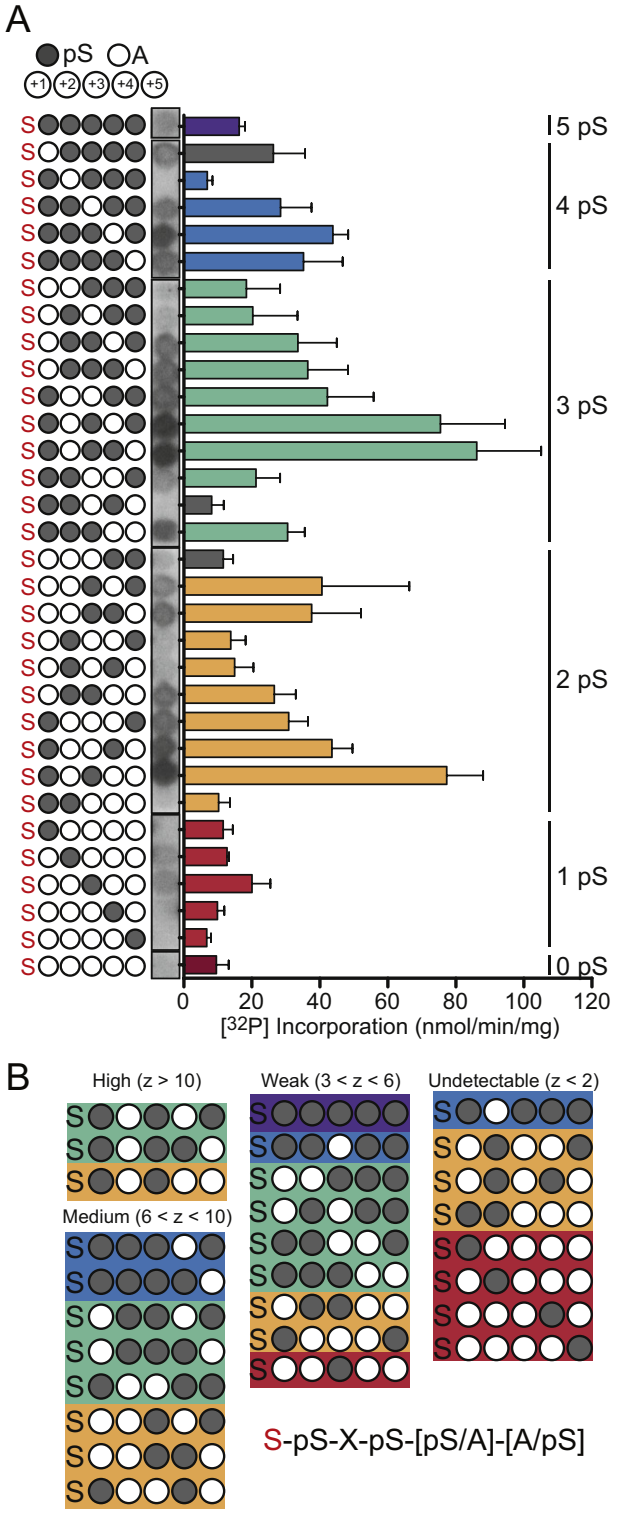

Fig. 1 – Hierarchical phosphorylation by CK2 requires the precise positioning of multiple phosphoserine residues. A. Biotinylated peptides based on the sequence Bi-WDDDSpSpSpSpSpSAAA, with specific phosphoserine residues systematically changed to alanine, were incubated with CK2 and [γ-^{32}P]-ATP. The phosphoacceptor residue in these sequences is shown in red. [γ-^{32}P] incorporation was visualized by autoradiography. Gray bars denote peptides that did not pass MS quality control. Average [γ-^{32}P] incorporation in three independent experiments is shown. Error bars indicate one standard deviation from the mean. B. Peptide sequences from A, sorted by z score (the number of standard deviations between each sample and a negative control (SAAAAA)) and colored based on number of phosphoserines. A z score greater than 1.95 corresponds to a P value of 0.05 or lower.

basic residues in the CK2 substrate binding pocket are optimally positioned to bind to acidic determinants at the +1 and +3 positions, and an additional two negative charges between these two binding sites may affect substrate binding dramatically. Moreover, phosphoserine, being bulkier than the acidic residues, may sterically alter the phosphorylation site, resulting in subtle structural changes that alter CK2 substrate binding. It is intriguing to speculate that this enhanced emphasis on the quantity and positioning of phosphodeterminants may act as a regulatory mechanism controlling these events, where the increased acidity of phosphoserine results in increased selectivity in the phosphorylation of primed substrates compared to canonical substrates. As sites of hyperphosphorylation are rampant in the human phosphoproteome [5], the strict requirements observed may ensure hierarchical phosphorylation by CK2 only at very specific layouts of phosphoserine residues.

In previous work on hierarchical phosphorylation by CK2, it was shown that while CK2 can use phosphoserine as an acidic determinant, it cannot use phosphothreonine in this manner [9]. However, this study was done with peptides containing only one phosphodeterminant (SAApSA, SAApTA), which in our results show only weak phosphorylation. Phosphotyrosine (pY) has also been demonstrated to act as a phosphodeterminant in CK2 hierarchical signaling [26], so it was included in our study as well. To compare phosphorylation of peptides containing each type of phosphodeterminant, we generated peptides corresponding to one of the most highly phosphorylated hierarchical peptides (SpSApSpSA) with phosphoserine substituted with either phosphotyrosine or phosphothreonine. Only phosphoserine proved efficient as a phosphodeterminant, with no detectable phosphorylation with phosphothreonine and very little with phosphotyrosine (Supplemental Fig. 1A). There are, however, anecdotal reports that CK2 may be able to utilize phosphothreonine or phosphotyrosine as phosphodeterminants in some sequence contexts. For example, CK2 phosphorylates a SYDE motif in CFTR, but only with prior tyrosine phosphorylation [27], and phosphorylates SLBP at T60, but only after prior phosphorylation of T61 [28]. These sites clearly have different spacing requirements than our data suggests for phosphoserine, suggesting that there may be additional CK2 hierarchical phosphorylation motifs, these requiring either phosphothreonine or phosphotyrosine at the +1 position, plus additional acidic determinants. At the phosphoacceptor site, even with identical phosphodeterminants to a highly phosphorylated serine-containing peptide, phosphorylation of threonine was only barely detectable (Supplemental Fig. 1B). It should also be noted that in certain cases, CK2 can phosphorylate tyrosine residues [29], but in the context of primed phosphorylation, this remains to be investigated. Taken together, these results show that optimal CK2 hierarchical phosphorylation occurs on serine residues, and is dependent on multiple phosphoserine residues with precise spacing.

3.2. CK2 canonical and hierarchical phosphorylation occur with comparable enzymatic efficiency

Initial screening identified a potential candidate motif for hierarchical phosphorylation by CK2, but for the phenomenon

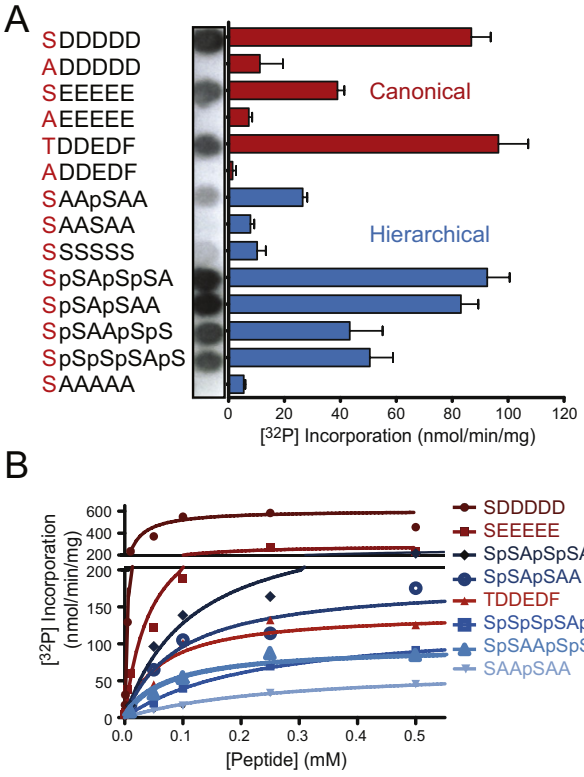

Fig. 2 – Hierarchical CK2 phosphorylation occurs with similar kinetics to canonical CK2 phosphorylation. A. Biotinylated synthetic peptides conforming to either canonical (red bars) or hierarchical (blue bars) consensus sequences were incubated with CK2 and [γ-^{32}P]-ATP before spotting onto streptavidin-coated membrane. The phosphoacceptor residue in these sequences is shown in red. [γ-^{32}P] incorporation was visualized by autoradiography. Values are the average of three independent experiments. Error bars indicate one standard deviation from the mean. B. Kinetic analysis of canonical (red lines) and hierarchical (blue lines) phosphorylation by CK2. Kinetic constants were determined by assaying each peptide for phosphorylation at a minimum of six different concentrations. Two graphs with different scales for the y-axis are used to present the data. Five replicates of each concentration were used in the analysis.

to be biologically relevant, it needs to be enzymatically favorable. When equal amounts of each peptide (0.5 mM) were incubated with CK2 and [γ-^{32}P]-ATP, the strongest of the hierarchical substrate peptides were phosphorylated at levels comparable to the optimal canonical peptides (WDDDSDDDDDAAA and WEEESEEEEEAAA) and a known canonical substrate of CK2, T1343 of Topoisomerase IIα [30], and at much higher levels than the previously published hierarchical peptide (SAApSAA) [9] (Fig. 2A). This suggests that hierarchical substrates of CK2 may be as efficiently phosphorylated by CK2 as canonical substrates. Canonical CK2 phosphorylation is widespread in the proteome [11], and regulates a number of important processes [10], so we next performed kinetic studies to compare the enzymatic efficiency of CK2 on select canonical and hierarchical peptides (Fig. 2B, Table 1). Km values of all three peptides modeled after

Table 1 – Kinetic analysis of canonical and hierarchical consensus sequences by CK2.

Peptide	Vmax (nmol/min/mg)	Km (μM)	Vmax/Km	SE_{Vmax}	SE_{Km}
WDDDSDDDDDAAA	608.8	16.74	36.37	22.01	2.592
WEEESEEEEEAAA	293	45.93	6.38	21.55	13.05
WDEKTDDEDFVPA	142.2	60.21	2.36	23.69	44.48
WDDDSAApSAAAAA	68.54	274.9	0.25	6.073	68.77
WDDDSpSApSpSAAAA	276.3	116.3	2.38	19.23	26.81
WDDDSpSApSAAAAA	183.4	91.13	2.01	16.59	31.37
WDDDSpSpSpSApSAAA	130.1	230.8	0.56	10.81	50.47
WDDDSpSAApSpSAAA	95.59	75.72	1.26	6.121	18.66

pS, phosphoserine; SE, standard error.

previously published peptides (SDDDDD, SEEEEE, and SAApSAA) followed the same trends and were similar to but lower than previously published Km values (60 μM, 18 μM, and 57 μM, respectively) [9,31]. As expected, the optimized canonical substrate (SDDDDD) was by far the best peptide in the study, with a Vmax/Km ratio (a measure of catalytic efficiency) of 36.37, compared to 6.38 for the SEEEEE peptide (demonstrating the difference in efficiency between aspartic acid and glutamic acid as determinants for phosphorylation), and 2.38 for the highest efficiency hierarchical peptide (SpSApSpSA). However, the peptide representing a known canonical mitotic substrate of CK2, T1343 of Topoisomerase IIα, despite having a lower Km value than any of the hierarchical substrates, provided a Vmax/Km value of 2.36, comparable to that of the SpSApSpSA peptide. Since T1343 of Topoisomerase IIα is effectively phosphorylated by CK2 *in vivo* [30], this suggests that given the correct sequence, CK2 may be just as likely to phosphorylate a hierarchical substrate as a canonical substrate.

Among the hierarchical substrate peptides, it appears that both too many and too few phosphoserines are detrimental to CK2 phosphorylation efficiency, as the peptides with one phosphoserine (SAApSAA) and four phosphoserines (SpSpSpSApS) have higher Km values than the peptides with two or three phosphoserines. This correlates well with the results in Fig. 1, where in general the highest CK2 activity was observed with diphosphopeptides and triphosphopeptides. The Km values of the most efficient hierarchical substrates (SpSApSpSA, SpSApSAA, and SpSAApSpS) were similar (116 nM, 91 nM and 76 nM respectively), and the relative efficiency of phosphorylation between these samples seemed to depend more on their Vmax values. Due to this, the hierarchical substrate with the lowest Km value (SpSAApSpS; 76 nM) was the least efficient substrate of the three, as the Vmax value for this peptide was two- to threefold lower than the others. These results indicate that it may be crucial to have the optimal number of phosphoserines in the correct spacing in order for a hierarchical substrate to have reaction kinetics as favorable as a canonical substrate.

3.3. *Determination of optimal consensus sequence for hierarchical CK2 phosphorylation*

To investigate whether additional amino acid residues might add specificity to sites of CK2 hierarchical phosphorylation, we used an oriented peptide array approach to further refine the consensus sequence. Since phosphoserines at the +1 and +3 positions were crucial for hierarchical phosphorylation, these sites were fixed as phosphoserine (parent peptide:WRRRAAASpSApSAAA). The alanine residues at the −3, −2, −1, +2, +4, +5, and +6 positions were systematically changed to other selected amino acids (D, E, F, H, K, M, N, P, Q, R, pS) and assayed for effects on CK2 hierarchical phosphorylation. Serine, threonine, and tyrosine were excluded to avoid the formation of additional phosphorylation sites, and tryptophan was excluded because it was used to quantitate peptide concentrations. We also excluded cysteine to avoid the formation of disulfide bonds between peptides, and small hydrophobic residues (G, I, L, and V) for redundancy with alanine.

Generally, substitution of alanine with basic residues was universally unfavorable, and the residue at the +2 position was crucial for phosphorylation, as the presence of any amino acid other than alanine, aspartic acid, or glutamic acid (and presumably glycine) blocked appreciable phosphorylation by CK2 (Fig. 3A). As with canonical CK2 phosphorylation, acidic residues were generally favorable, particularly at the −1 and +4 positions. Phosphoserine, however, was not favorable at the −1 or +4 position, suggesting that acidic and phosphorylated residues are not completely interchangeable as consensus determinants. Phosphoserine did increase phosphorylation when positioned further away from the phosphoacceptor site, at the −3, −2, +5, or +6 position. We also observed a slight increase in phosphorylation when either histidine or phenylalanine was present at the −3, −2, or −1 position. In addition, histidine at the +4 position increased CK2 hierarchical phosphorylation. Interestingly, neither histidine nor phenylalanine affects canonical CK2 phosphorylation [14].

As acidic residues are also crucial for CK2 canonical phosphorylation, we wanted to determine if D/E themselves were primarily driving phosphorylation, or if they combined with phosphoserine to generate a stronger motif. Dephosphorylation of D/E-containing phosphopeptides with lambda phosphatase before incubation with CK2 results in a complete loss of phosphorylation (Fig. 3B), supporting the notion that the acidic residues are acting as positive determinants for hierarchical phosphorylation. Taken together, the results indicate that the ideal substrate consensus sequence for CK2 hierarchical phosphorylation is not only multiply phosphorylated, but also contains additional acidic residues (Fig. 3C). The positive impact of acidic residues on CK2 hierarchical

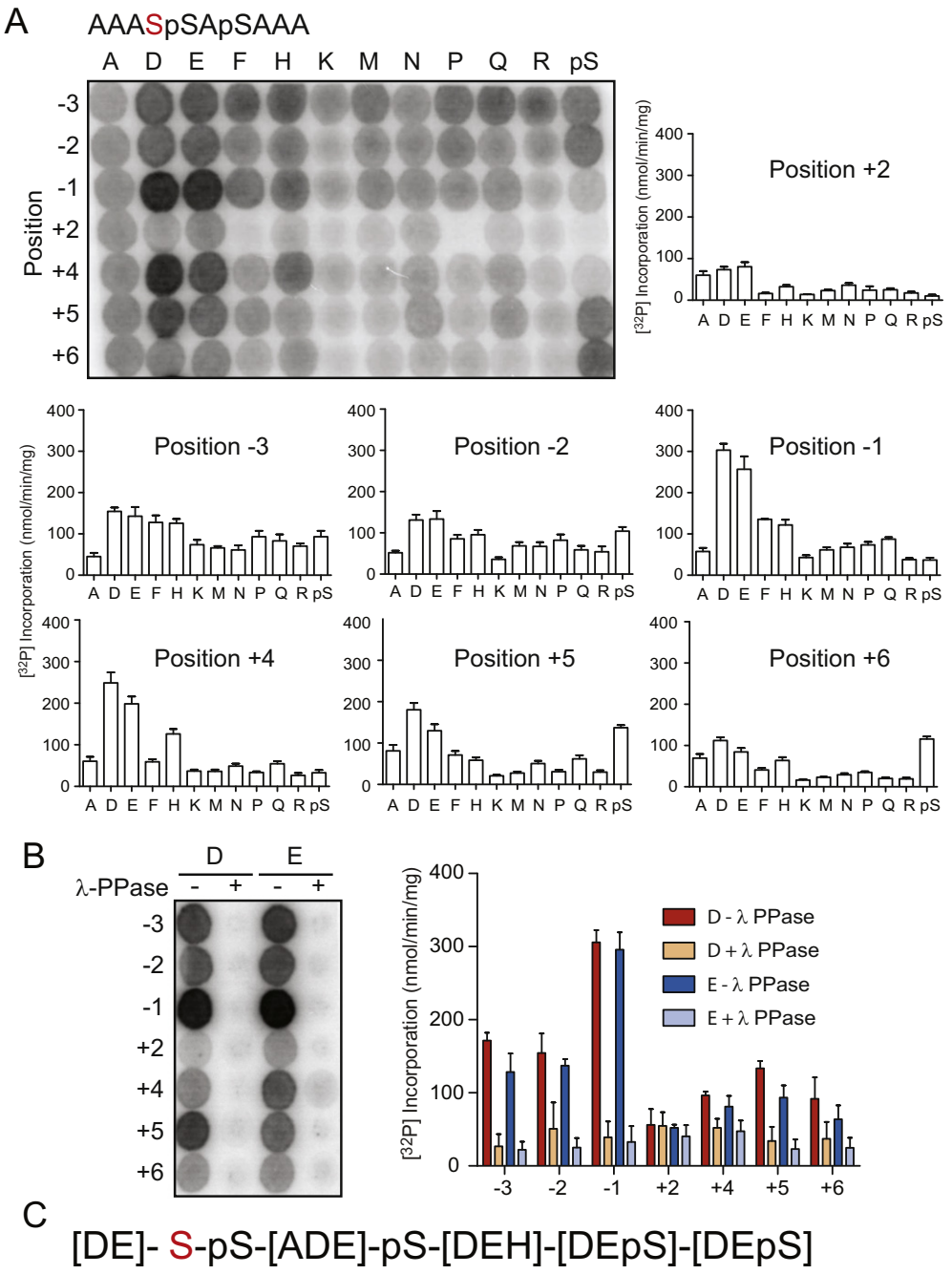

Fig. 3 – Determination of optimal consensus sequence for primed CK2 phosphorylation. A. Synthetic peptides based on the sequence WRRRAAASpSApSAAA, with alanines systematically changed to various amino acid residues, were incubated with CK2 and [γ-^{32}P]-ATP. Average [γ-^{32}P] incorporation into each peptide is also shown. All values are the average from three independent experiments. Error bars indicate one standard deviation from the mean. B. Peptides containing D/E at each position were treated with λ-phosphatase before incubation with CK2 as above. C. Optimal consensus sequence for CK2 hierarchical phosphorylation.

phosphorylation suggests that *in vivo*, canonical and hierarchical phosphorylation consensus sequences probably exist in a continuum, with consensus elements from both combining to form a functional phosphorylation site. In this manner, the extent of CK2 phosphorylation could be fine-tuned for each individual substrate at the level of the consensus sequence.

3.4. *Proline-directed kinases can combine with canonical determinants to generate CK2 hierarchical phosphorylation motifs*

The optimal consensus sequence for CK2 hierarchical phosphorylation, as outlined above, provides guidelines on how CK2 might use multiple phosphoserine residues to facilitate upstream phosphorylation. However, these motifs

could represent sites of processive multisite phosphorylation by CK2 alone (provided that the initial priming phosphoserine was itself canonically phosphorylated). While the existence of such sites would, in itself, open up new avenues for phosphoregulation by CK2, the possibility of CK2 using phosphorylation sites catalyzed by distinct kinases as phosphodeterminants is even more intriguing.

CK2 is a somewhat puzzling kinase — it is constitutively active and ubiquitously expressed, yet participates in a number of exquisitely regulated biological processes [10]. Hierarchical phosphorylation by CK2, primed by the activation of a regulated kinase, could explain in part how this regulation is possible. For example, cyclin-dependent kinases (CDKs), a family of proline-directed serine/threonine kinases, are tightly regulated enzymes, controlled by intricate signaling pathways that ensure activation only during the appropriate phase or stimulus [32]. CDKs control several important cellular processes, including cell cycle progression and transcriptional regulation [33], processes which also require regulated CK2 activity [10]. Importantly, due to the inhibitory effects of proline at the +1 position, CK2 is highly unlikely to phosphorylate a proline-directed serine residue [12], and would therefore be unable to phosphorylate these motifs in a processive manner. Therefore, to investigate the possibility of non-processive (primed) hierarchical phosphorylation by CK2, we explored the possibility of hierarchical phosphorylation by CK2, primed by a proline-directed kinase. Since CK2 hierarchical phosphorylation favors a +3 phosphoserine, we chose to position a generic CDK consensus site, SPVK [34], with the CDK-targeted serine at the +3 position. To determine the consensus requirements for CK2 hierarchical phosphorylation primed by a CDK, we based our assay on the peptide WDDDSAApSPVKAA, with the proline-directed phosphoserine at the +3 position, and changed either the +1 or +2 amino acid to arginine, phenylalanine, methionine, glutamine, histidine, phosphoserine, proline, or aspartic acid. Upon incubation with CK2 and [γ^{32}P]-ATP, even the alanine containing control peptide showed minimal phosphorylation by CK2 (Fig. 4A). Aspartic acid at the +1

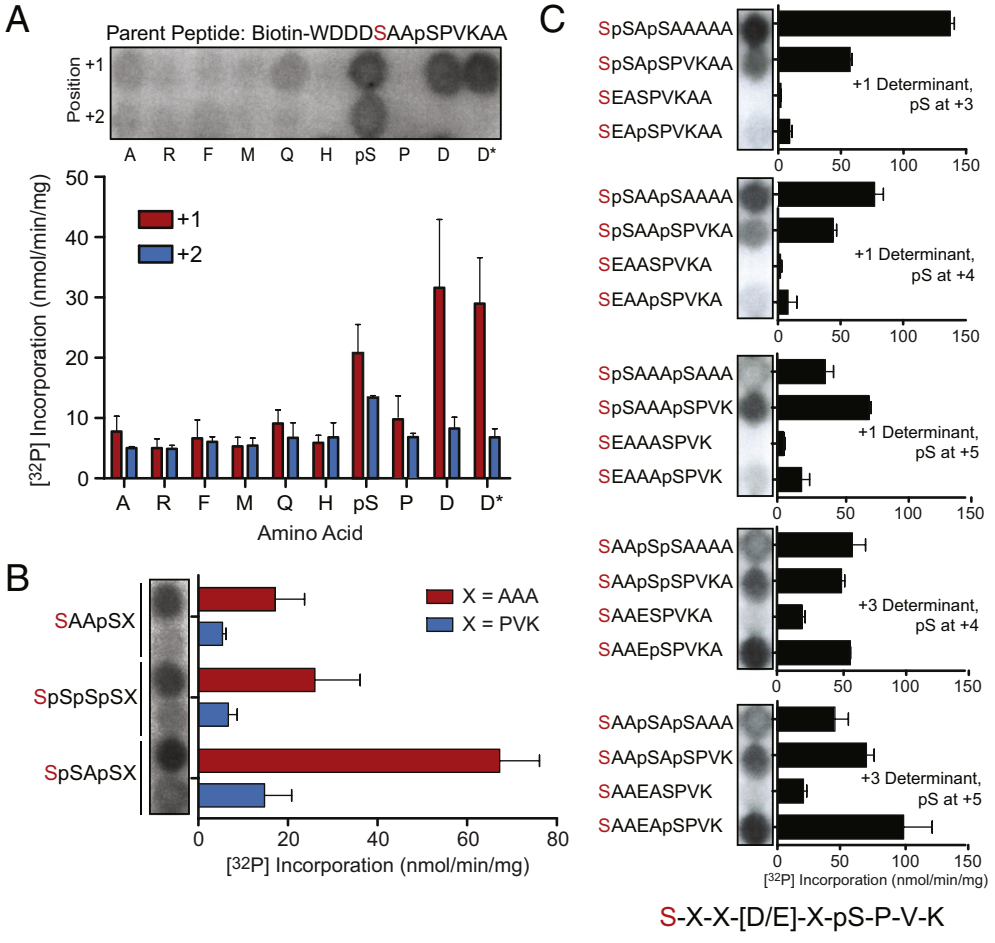

Fig. 4 – Proline-directed hierarchical phosphorylation requires a mix of canonical and primed CK2 consensus elements. A. Starting from the parent peptide Bi-WDDDSAApSPVKAA (phosphoacceptor residue in red), the +1 and +2 positions were changed to various amino acids, including phosphoserine (D*: D-containing peptide with unphosphorylated serine at priming site). B. Peptides with similar CK2 determinants, with or without a downstream proline-directed site, were assayed for phosphorylation by CK2. C. Biotinylated peptides combining glutamic acid at +1 or +3 with a P-directed site at +4, +5, or +6 were assayed for CK2 phosphorylation. Values are an average of three independent experiments. Error bars indicate one standard deviation from the mean.

position increased phosphorylation substantially; however, this seems to be solely due to the formation of a canonical CK2 site, as an unphosphorylated control peptide showed similar levels of [γ^{32}P] incorporation (compare the rightmost columns of Fig. 4A). In agreement with known canonical requirements, aspartic acid at the +2 position did not increase CK2 phosphorylation. The only peptides hierarchically phosphorylated in the assay were those with an additional phosphoserine at either the +1 or +2 position, indicating that, as in the canonical consensus, multiple phosphorylated residues may be required for appreciable hierarchical phosphorylation. The lack of kinase activity towards the peptides in Fig. 4A suggested that a +3 phosphodeterminant was not optimal for proline-directed CK2 hierarchical phosphorylation, as the presence of the CDK consensus site seemed to inhibit CK2 phosphorylation. To confirm this, we compared the phosphorylation of peptides containing identical consensus determinants with either a string of alanine residues (AAAAA) or a Cdk1 consensus site (PVKAA) following the consensus phosphoserines. These peptides clearly show that CDK consensus determinants severely impair the ability of CK2 to phosphorylate primed sequences when present at the +4 to +6 positions relative to the CK2 site (Fig. 4B).

Our results suggest that hierarchical phosphorylation by CK2 requires multiple priming phosphodeterminants, and can use acidic determinants in combination with phosphodeterminants. Since hierarchical phosphorylation by a proline-directed kinase and CK2 would presumably occur with only a single phosphorylated residue, and is inhibited by close proximity to CDK consensus determinants, we wondered if CDK phosphorylation farther upstream could synergize with existing canonical determinants, providing enough additional acidity to the region to form a novel CK2 site. Since aspartic acid is a much stronger determinant for CK2 phosphorylation than glutamic acid [31] (also compare the SDDDDD and SEEEEE peptides in Fig. 2), we used glutamic acid to investigate the effects of addition of a proline-directed phosphoserine to a weak canonical CK2 site. To do this, we generated biotinylated peptides with a CDK site phosphoserine at the +3, +4, or +5, and glutamic acid at either the +1 or +3 position, and tested the phosphorylation of these peptides by CK2. We compared primed peptides with either alanine residues or a CDK consensus sequence following the priming phosphoserine, and interestingly, while all other primed peptide pairs tested in this study demonstrated a sharp decrease in phosphorylation upon addition of the CDK determinants (Fig. 4B), a CDK site at the +5 position displayed increased phosphorylation compared to alanine residues (Fig. 4C, compare the first two peptides of each panel). This suggests that, while unfavorable when directly adjacent to the CK2 site, the prolyl, hydrophobic, and basic determinants comprising a CDK consensus sequence may actually become favorable determinants for CK2 phosphorylation when situated farther away from the CK2 phosphoacceptor residue. When the phosphodeterminant at +1 or +3 was replaced with glutamic acid, peptides containing one glutamic acid and an unphosphorylated CDK site were barely phosphorylated; indicating that these sequences represent very weak canonical CK2 sites (Fig. 4C, third row of each panel). Glutamic acid at the +1 position combined with a proline-directed phosphoserine was insufficient for phosphorylation to occur. However, glutamic acid at the +3 position combined with a proline-directed phosphoserine resulted in a significant increase in CK2 phosphorylation compared to the unphosphorylated peptide, with a CDK site at the +5 position particularly favorable. Based on these results, we conclude that proline-directed hierarchical phosphorylation by CK2 would require both canonical and primed consensus determinants, and would occur optimally at sequences with a canonical determinant at the +3 position followed by a proline-directed phosphorylation site at the +5 position (S-X-X-D/E-X-pS-P).

3.5. *Candidate substrates for CK2 hierarchical phosphorylation are enriched for regulators of several important cellular signaling pathways*

Thus far, we have outlined optimal consensus sequences for hierarchical phosphorylation by CK2, and demonstrated that the phenomenon is kinetically favorable. We next sought to determine if sites matching the consensus sequences existed in the human proteome. To achieve this objective, we searched the NCBI non-redundant human proteome to identify human proteins containing peptide sequences matching either the multisite hierarchical sequence (SS[ADEG]S[ADEGLIVFHNQ][ADEGLIVFHNQS][ADEGLIVHS]; Supplemental Table S2), or the optimal spacing and consensus determinants for proline-directed hierarchical phosphorylation (S[DE]X[DE]XSP and S[ACDEFGHILMNQSTVWY]X[DE]XSP, where X is any amino acid; Supplemental Table S3). Importantly, the sequences used in the peptide match search do not match the optimal spacing of consensus determinants for phosphorylation by CK1 (pS/pTXXS) [8] or GSK3 (SXXXpS) [7], suggesting that no other known hierarchical kinase is likely to appreciably phosphorylate these sites *in vivo*. Based on these searches, 1319 amino acid motifs matching the CK2 multisite hierarchical phosphorylation consensus sequence were found in the proteome. These sites were contained within 1123 proteins, with almost 15% of the proteins containing more than one potential motif. The searches also revealed 552 proline-directed CK2 hierarchical phosphorylation motifs in 523 proteins, with over 5% of proteins containing more than one consensus motif. According to the PhosphoSitePlus [3] phosphorylation site database, 14.18% of putative multisite motifs and 28.44% of proline-directed motifs contain at least one

Table 2 – Sequences in the human proteome matching CK2 hierarchical phosphorylation consensus requirements.

	Multisite	Proline-directed
Matching consensus sequences in proteome	1319	552
Proteins containing matching sequence(s)	1123	523
% proteins containing multiple sequences	14.86	5.25
Consensus sequences with known phosphorylation sites	187	157
% consensus sequences with known phosphorylation sites	14.18	28.44
Consensus sequences with all sites phosphorylated	61	50
% consensus sequences with all sites phosphorylated	4.6	9.1

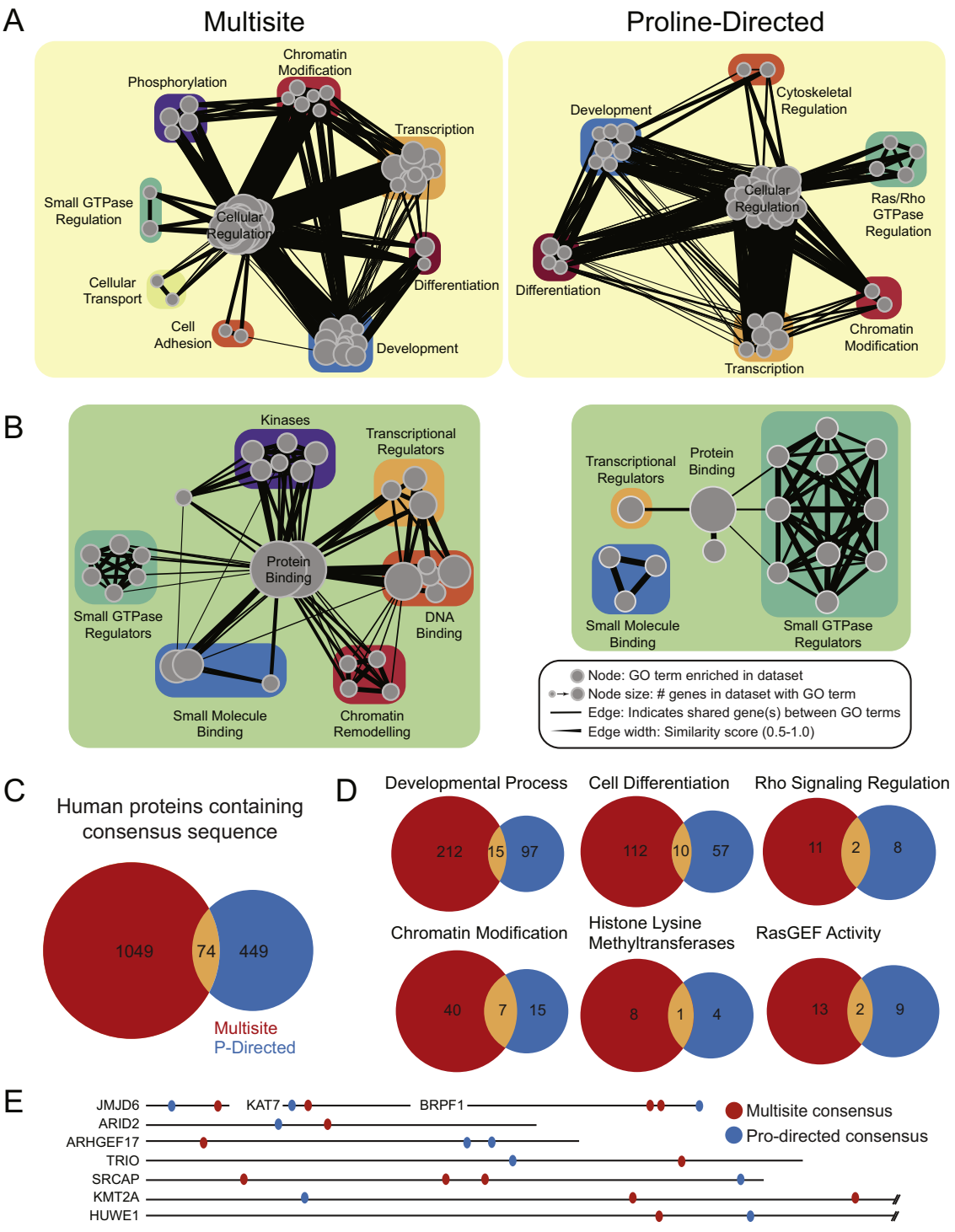

Fig. 5 – CK2 hierarchical consensus motifs are enriched in genes involved in a specific subset of cellular processes. The NCBI non-redundant human proteome was searched for genes with sequences matching either the multisite consensus (SS[ADEG]S[ADEGLIVFHNQ][ADEGLIVFHNQS][ADEGLIVHS]) or proline-directed consensus (S[DE]X[DE]XSP and S[ACDEFGHILMNQSTVWY]X[DE]XSP, where X is any amino acid). Resulting gene sets were analyzed for enriched biological processes (A) and enriched molecular functions (B) by Gene Ontology using DAVID. Enrichment map was used to visualize the enrichment data, with a P value cutoff of 0.005 and an FDR cutoff of 0.1. C. Protein overlap between complete multisite and Pro-directed datasets, and D, within enriched functional groups. E. Location of multisite and pro-directed motifs in selected proteins containing both motifs.

previously reported *in vivo* phosphorylation site (Table 2; Supplemental Tables S2 & S3). In addition, 61 proteins with multisite motifs are phosphorylated *in vivo* at all three sites, and 50 proteins with proline-directed motifs have been reported to be phosphorylated at both the proline-directed and hierarchical sites. Gene ontology analysis of the predicted CK2 hierarchical

consensus motifs suggests that the occurrence of these motifs in the proteome is not entirely random. In this respect, functional enrichment analysis of the peptide match datasets produced several distinct and highly significant clusters, indicating that hierarchical phosphorylation by CK2 may occur specifically in certain biological pathways (Fig. 5A, B, Supplemental Tables S2 and S3).

Several crucial biological processes were enriched for proteins containing either consensus motif, including control of gene expression, and small GTPase signaling. Regulators of gene expression included both direct transcriptional regulators (including a total of 129 transcription factors with either motif), as well as proteins regulating chromatin modification (69 total proteins). Canonical CK2 phosphorylation is a well-known regulator of transcription, directly regulating RNA polymerase II via C-terminal domain phosphorylation. Furthermore, CK2 phosphorylation at canonical motifs has also been demonstrated for several RNA polymerase II general transcription factors, including TFIIA, TFIIE, and TFIIF [35] as well as a variety of other transcription factors, leading to either activation or repression of transcription, depending on the substrate [10]. The functional enrichment observed for hierarchical sites in proteins regulating gene expression suggests that the effects of CK2 on transcriptional regulation may be currently understated. Another area of strong enrichment in both datasets was in regulators of small GTPase-mediated signal transduction, particularly involving Ras and Rho signaling pathways, which are responsible for regulating proliferation and actin cytoskeleton dynamics, respectively [36]. In particular, guanine exchange factors (GEFs), which help the GTPase exchange GDP for GTP [36], were highly enriched, with 15 RasGEFs and 13 RhoGEFs containing a multisite motif, and 11 RasGEFs and 10 RhoGEFs containing a proline-directed motif. Although CK2 has well known functions in the regulation of both proliferation [10] and actin dynamics [37], and plays a role in Ras-mediated transformation [38,39], we found no reported evidence of a direct regulatory role for CK2 in either Ras or Rho function, suggesting that this may represent a novel role for CK2.

Strikingly, despite the lack of overlap between the multisite (SSXS) and proline-directed (SXX[D/E]XSP) consensus sequences, the motifs display largely overlapping enrichment patterns. Generally, the overlap in functional enrichment cannot be attributed to the presence of motifs for multisite and proline-directed hierarchical phosphorylation occurring in the same proteins, as a minimal overlap exists between the two datasets (Fig. 5C). Even within functionally enriched groups, very little overlap exists between proteins that have multisite hierarchical motifs and proteins that have proline-directed motifs (Fig. 5D). The overlap that does exist, however, represents a small subset of proteins that do indeed contain both types of motif. For example, both datasets are enriched for histone lysine methyltransferases, but the only individual enzyme in both datasets is KMT2A, which contains one proline-directed motif and two multisite motifs (Fig. 5E).

We also observed significant enrichment of several conserved domains in proteins containing hierarchical phosphorylation motifs, including several protein- and DNA-binding motifs important in the enriched biological processes. For example, multisite phosphorylation motifs were often found in proteins containing domains important for transcriptional regulation, including bromodomains, homeobox domains, and zinc fingers (Fig. 6A), while proline-directed motifs occurred in proteins containing domains involved in signal transduction, including protein–protein binding domains (SH3, PDZ), and RhoGEF (DH) domains (Fig. 6B). For the most part, enriched conserved domains were specific to one type of hierarchical phosphorylation. This suggests that not only is the striking overlap in functional enrichment not due to protein overlap between the two motifs (Fig. 5C), but it is also not due to a functional domain overlap (compare Figs. 6A and B). As demonstrated for several groups of proteins containing enriched domains as well as one or more hierarchical phosphorylation motifs (Fig. 6C, D), in the majority of cases, hierarchical phosphorylation motifs are positioned outside the enriched domains. Therefore, the observed domain enrichment was not due to the hierarchical phosphorylation motif being contained within the conserved domain. While in some cases the hierarchical motif is quite close to an enriched conserved domain (for example, the SH3-adjacent motifs in MAST1-4 (Fig. 6D)), for the most part there seems to be no real pattern governing the positioning of the motifs relative to any conserved domains, particularly among more divergent proteins (compare the MAST proteins to the SNF2 containing proteins (Fig. 6C) and RhoGEF-containing proteins (Fig. 6D)). It is tempting to speculate that CK2 hierarchical phosphorylation motifs may have co-evolved with these conserved domains to regulate biological processes. Generally, motifs for hierarchical phosphorylation by CK2 seem to be restricted to proteins involved in the regulation of specific processes. The two phosphorylation motifs appear independent of each other, and yet seem to regulate the same specific processes, through the regulation of different proteins with different conserved functional domains.

The enrichment analysis demonstrates that potential CK2 hierarchical phosphorylation motifs are not randomly distributed in the proteome, and are significantly associated with specific cellular processes, functions, and conserved domains. This raises the intriguing prospect that hierarchical phosphorylation by CK2 could have a significant impact on signal transduction. The majority of known phosphorylation sites matching hierarchical motifs have not been characterized for biological function. However, one of the many transcription factors containing a multisite motif, CTCF, is a known substrate of CK2 *in vitro* and *in vivo*, with well-documented phosphorylation at S609, S610, and S612 [40,41], despite somewhat weak canonical phosphodeterminants. While located outside the zinc fingers, phosphorylation at all three sites is crucial for the modulation of c-Myc transcription, and therefore, cell proliferation. Therefore, these phosphorylation sites may represent an example of CK2 hierarchical phosphorylation with a significant impact on cellular signaling. It will be crucial to validate additional sites of hierarchical phosphorylation in the proteome, with emphasis on determining the functional roles of phosphorylation at these motifs.

Typically, regulated CK2 activity towards a particular substrate has been attributed to changes in subcellular localization,

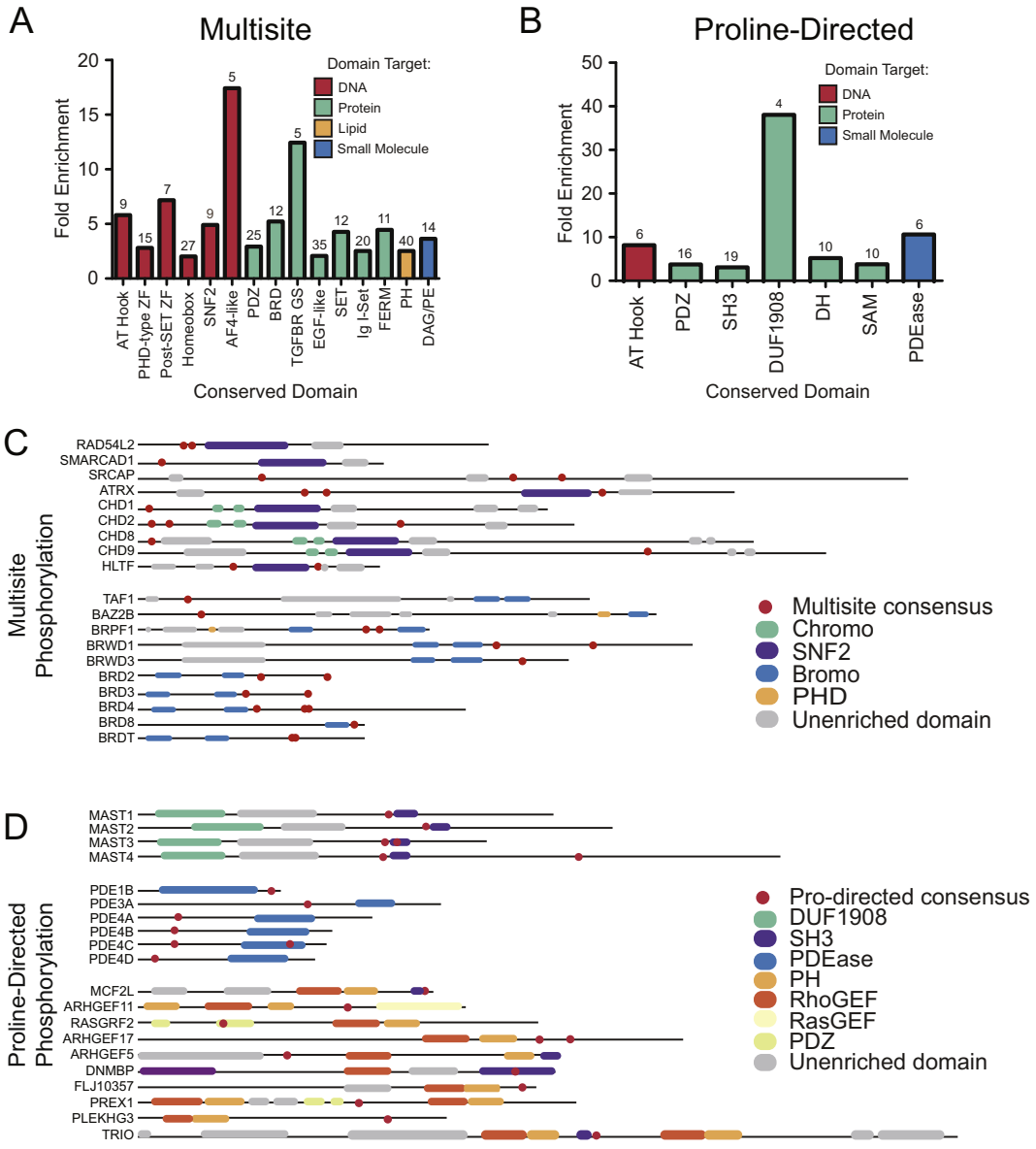

Fig. 6 – Putative CK2 hierarchical phosphorylation sites are enriched in genes containing specific conserved domains, but do not occur within the domains. A, B. Enriched conserved domains in genes containing multisite (A) and proline-directed (B) CK2 hierarchical consensus matches. FDR < 0.05. C, D. Schematic representations of genes containing multisite (A) and proline-directed (B) CK2 hierarchical consensus matches along with conserved domains.

protein–protein interactions, and other more subtle means. Hierarchical phosphorylation adds another facet to CK2 regulation, at the level of the substrate consensus sequence. The ability to add and remove various phosphodeterminants gives hierarchical consensus sequences flexibility not found with genetically-encoded kinase consensus determinants. In the case of multisite hierarchical phosphorylation, the precise phosphodeterminants available to CK2 could change under various stimuli, leading to subtle modulation of the level of CK2 phosphorylation at these sites. The added regulation becomes even clearer in the case of hierarchical phosphorylation requiring a distinct priming kinase, as in the case of the proline-directed sites. Due to the regulated activity of the priming kinase, proline-directed hierarchical phosphorylation would place CK2, a constitutively active kinase, under the same strict regulation at these sites, with widespread canonical CK2 phosphorylation unaffected. This may explain how CK2 can act in tightly regulated processes.

The results of this study suggest that the impact of hierarchical phosphorylation on cellular signaling may be extensive, and adds considerably to the already long list of potential CK2 substrates. The functional effects of these modules may depend on the extent of phosphorylation within the region, as well as the pattern of the phosphorylation sites [42]. For example, the pattern and magnitude of phosphorylation of a cluster could provide subtle modulations in protein structure. It is also tempting to speculate that hierarchical phosphorylation of clusters of sites could also be more resistant to dephosphorylation than single sites. The extreme clustering of phosphorylation sites observed in the phosphoproteome had

led to the notion that clusters of phosphorylation sites may collectively act as higher order modules that act as one to elicit a certain biological response [4]. Adding more complexity, it is likely that phosphorylation events, combined with additional post-translational modifications such as acetylation and ubiquitylation, may form distinct signaling modules, much like the histone code that governs chromatin organization [43]. As databases reporting *in vivo* post-translational modifications continue to grow, it will become increasingly crucial to understand the combinatorial effects that these modifications have on proteins and signaling pathways.

4. Conclusions

In this study, we have investigated the possibility that hierarchical phosphorylation by CK2 may play a role in cellular signal transduction. Hierarchical phosphorylation by CK2 requires multiple consensus determinants with specific spacing, and can be as enzymatically efficient as canonical phosphorylation. Consensus motifs are found in a variety of human proteins, several of which are known to be multiply phosphorylated in cells at the corresponding sites. Potential substrates of CK2 hierarchical phosphorylation were significantly enriched among regulators of several crucial processes, including gene expression, development, and small GTPase signaling. Taken together, our results provide strong *in vitro* evidence that hierarchical phosphorylation by CK2 may have several important roles in cellular signaling pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.10.020.

6. Transparency document

The Transparency document associated with this article can be found, in the online version.

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