

Phosphorylation in Protein-Protein Binding: Effect on Stability and Function

Hafumi Nishi,¹ Kosuke Hashimoto,¹ and Anna R. Panchenko^{1,*}

¹National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20892, USA

*Correspondence: panch@ncbi.nlm.nih.gov

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SUMMARY

Posttranslational modifications offer a dynamic way to regulate protein activity, subcellular localization, and stability. Here we estimate the effect of phosphorylation on protein binding and function for different types of complexes from human proteome. We find that phosphorylation sites tend to be located on binding interfaces in heterooligomeric and weak transient homooligomeric complexes. Analysis of molecular mechanisms of phosphorylation shows that phosphorylation may modulate the strength of interactions directly on interfaces and that binding hotspots tend to be phosphorylated in heterooligomers. Although the majority of complexes do not show significant estimated stability differences upon phosphorylation or dephosphorylation, for about one-third of all complexes it causes relatively large changes in binding energy. We discuss the cases where phosphorylation mediates the complex formation and regulates the function. We show that phosphorylation sites are more likely to be evolutionarily conserved than other interfacial residues.

INTRODUCTION

Cellular regulatory mechanisms provide a sensitive, specific and robust response to external stimuli and posttranslational modifications offer a dynamic way to regulate protein activity, subcellular localization, and stability (Olsen et al., 2006; Ptacek and Snyder, 2006; Schlessinger, 2000). Such dynamic regulation is achieved through reversibility and fast kinetics of posttranslational modifications, such as when, for example, a phosphate group can be quickly attached and removed by kinases and phosphatases, respectively. Indeed, adding or removing a dianionic phosphate group somewhere on a protein might change its physico-chemical properties, stability, kinetics, and dynamics (Johnson, 2009). Recent phosphoproteomic analyses have revealed that the majority of proteins in a mammalian cell are phosphorylated (Olsen et al., 2006; Olsen et al., 2010), so regulatory mechanisms involving phosphorylation are very widespread.

Many signaling and other types of pathways involve a dense network of protein-protein interactions, and the reaction rates of these processes, among other factors, will depend on protein concentrations and association/dissociation constants

of protein assemblies. Phosphorylation can be used to modulate the nature and the strength of protein-protein interactions, thereby regulating protein binding and coordinating different pathways. If phosphorylation occurs at or near a binding interface, it may directly affect the binding energy of the complex. At the same time, phosphorylation of a site outside a binding interface may cause long-range conformational changes through allosteric mechanisms and affect the binding of the partner, as observed for the classical example of glycogen phosphorylase (Jenal and Galperin, 2009; Lin et al., 1997). Another aspect of coupling between phosphorylation and binding is the recognition of the phosphates by special phospho-Ser/Thr or Tyr binding domains (such as 14-3-3, SH2, MH2, and others); such a process may release the protein from autoinhibition and result in activation and subsequent signal propagation, as in the case of Src kinases (Schlessinger, 2000). Finally it has been shown that flexible regions and intrinsically disordered proteins have a tendency to be phosphorylated, and phosphorylation might induce disorder-to-order as well as order-to-disorder transitions (Antz et al., 1999; Collins et al., 2008; Gsponer et al., 2008; Radhakrishnan et al., 1997).

In this article, we analyze the effect of phosphorylation on protein binding for different types of complexes from the human proteome varying by stability and the nature of the interacting subunits. We show that there exists a coupling between phosphorylation and protein-protein binding for all types of heterooligomeric and weak transient homooligomeric complexes. Computational alanine scanning experiments and analysis of the energetic effect of attaching/removing phosphate groups show that phosphorylation may modulate the strength of interactions directly on interfaces and that binding hotspots have a tendency to be phosphorylated for heterooligomers. Although for many Ser/Thr/Tyr sites we did not find significant stability differences upon attaching/removing the phosphate group, for one-third of all complexes this brings about a relatively large change in binding energy (more than 2 kcal/mol). We analyze the effect of phosphorylation on protein function and show that several pathways, especially the hemostasis pathway, are enriched with phosphoproteins and phosphosites. Finally, we show that phosphosites on interfaces are more likely to be evolutionarily conserved than other interfacial residues.

RESULTS

Coupling between Phosphorylation and Protein-Protein Binding

Using a nonredundant set of 933 structures of phosphorylated human hetero- and homooligomeric complexes (see

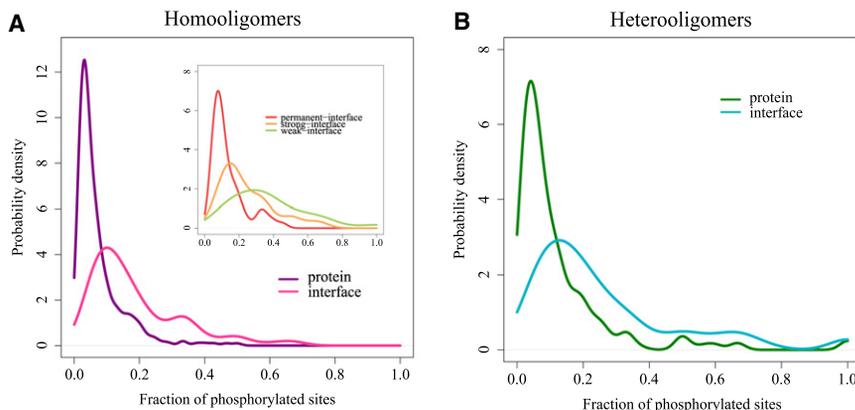


Figure 1. Probability Density Function of the Fraction of Phosphosites in Protein Complexes and on Binding Interfaces for Homooligomers and Heterooligomers

Homooligomers (A) had 308 phosphosites in proteins and 111 on interfaces, and heterooligomers (B) had 290 phosphosites in proteins and 160 on interfaces. The difference between the mean values of these distributions is significant (p value = $2e-16$ for both homooligomers and heterooligomers by Wilcoxon rank-sum test). Inset: curves for permanent homooligomers are shown in red, strong transient homooligomers are shown in orange, and weak transient homooligomers are shown in green. The distributions are smoothed by the Gaussian kernel density estimation. See also Tables S1 and S2.

Experimental Procedures for detail), we observed on average two phosphorylation sites (pTyr, pSer, or pThr) per protein. Note that the majority of protein complexes do not have actual phosphate groups in the Protein Data Bank (PDB) structures (Zanzoni et al., 2011). As one can see from Figure 1, the distribution of fractions of phosphosites in phosphoproteins is quite narrow with a large majority of all phosphocomplexes having about 5%-10% of all Ser, Thr, or Tyr residues phosphorylated. The distribution has a long tail, however, which is consistent with the fact that proteins with multiple phosphorylation sites occur more often than expected by chance, in agreement with previous studies for *Arabidopsis thaliana* (Riaño-Pachón et al., 2010). Overall, we observed the relative fractions of the types of phosphosites to be ~40% pSer, ~25% pThr, and ~35% pTyr in protein structural complexes, and this observation did not depend on whether the complexes represented hetero- or homooligomers. The frequencies of pSer, pThr, and pTyr observed in structural complexes were quite different from those obtained in high-throughput experiments for phosphoproteomes, which identified only a small fraction of pTyr sites (Hunter and Sefton, 1980; Olsen et al., 2006). This discrepancy may be explained by the observation that hydrophobic Tyr is more likely to be found in structured regions, whereas Ser and Thr are frequently found in disordered and flexible regions. Indeed, it was reported recently that almost half of pTyr sites were located within conserved protein domains (Sugiyama et al., 2008). Moreover, tyrosine phosphorylation might occur on less abundant proteins compared to serine and threonine phosphorylation, hence the statistics for rather redundant phosphoproteomes may differ from our nonredundant set.

Further, we studied the coupling between phosphorylation and protein-protein binding by examining binding interfaces and locations of phosphosites in complexes (see Table S1 available online). Overall, we found that the association between phosphorylation sites and binding interfaces is very strong for heterooligomers (Fisher's exact test, p value = $7.4e-15$) and significant but not so prominent for weak transient homooligomers (p value = 0.0008) (Figure 1; Table 1; Table S2). No association was found for permanent and strong transient homooligomers. Because the stability of the complex depends on the number of subunits, we also performed a similar analysis restricted to dimers and found a similar trend (p value = $5.3e-06$

for heterooligomers). The tendency of phosphosites to be involved in binding did not correlate with the estimated stability of heterooligomers, which in turn were generally less stable than all homooligomers according to our analysis (Wilcoxon rank-sum test, p value = 0.03). These results are consistent with our previous study, which showed that transient complexes that bind different protein partners using the same interface (promiscuous binding) are enriched with Tyr, Ser, and Thr (among a few other residues) on their interfaces, and their phosphorylation may provide the switch between different functional pathways (Tyagi et al., 2009).

Structural Environment of Phosphorylation Sites

Although phosphorylation sites are usually located on protein surfaces, some of their structural properties are different from the other surface residues (Gnad et al., 2007; Jiménez et al., 2007; Zanzoni et al., 2011). We analyzed the structural properties of phosphosites (sites that can be phosphorylated even if there is no actual phosphate present in the PDB structure) on interfaces to see if these properties are different from nonphosphorylated Ser/Thr/Tyr sites on interfaces. Phosphosites in heterooligomers seem to be more solvent accessible than non-phosphorylation sites in isolated protomers (on average by 23 \AA^2 ; p value = $2.2e-16$) and tend to change solvent accessibility upon complex formation by burying more surface area (on average by 13 \AA^2 ; p value = $2.2e-16$, Table 1; Figure S1). This is consistent with our previous observation that phosphosites are predominantly located on binding interfaces. At interfaces, phosphorylation sites contribute to the complex stability by forming more hydrogen bonds and residue contacts than non-phosphosites (for hydrogen bond difference, p value = 0.0005 for heterooligomers and p value = 0.04 for weak homooligomers; Table 1). Additionally, Tyr residues tend to be located in the core of protein interfaces, playing a critical role for oligomerization through aromatic stacking interactions, its phosphorylation therefore might directly affect the binding affinity. The estimate of binding energy provides additional evidence for these findings, as shown in the following section.

Energetic Effect of Phosphorylation

Residues that are essential for the structural integrity of proteins or protein complexes are called binding hotspots (Bogan and

Table 1. Properties of Phosphorylation Sites on Protein Binding Interfaces

	All	All Heterooligomers	All Homooligomers	Homooligomers		
				Weak	Strong	Permanent
Abundance on interface	1.5e-13*	7.4e-15*	0.097	8.2e-04*	0.417	0.054
Structural properties						
Protomer ASA ^a	2.2e-16*	2.2e-16*	0.065	0.057	0.318	0.137
Δ ASA ^b	4.5e-08*	1.7e-09*	0.482	0.609	0.361	0.050
No. of hydrogen bonds per site	5.9e-05*	5.3e-04*	0.043*	0.042*	0.272	0.202
No. of residue-residue contacts per site	2.8e-04*	1.1e-03*	0.217	0.502	0.424	0.452
Energetic properties ^c						
$\Delta\Delta\Delta G^{\text{Ala}}$	1.8e-03*	2.7e-04*	0.494	0.181	0.670	0.390
$\Delta\Delta\Delta G^{\text{P}}$	2.2e-16*	1.3e-12*	1.3e-08*	2.8e-05*	1.1e-04*	1.6e-05*
Evolutionary conservation of site	0.018*	0.016*	0.296	0.558	0.113	0.654

All values are presented as p values. The “Abundance on interface” row presents p values calculated by Fisher’s exact test showing association between being phosphorylated and location on binding interface (compared to surface). All other rows present p values calculated by Wilcoxon rank-sum test showing the difference between phosphosites and nonphosphosites on binding interfaces with respect to different properties. Significant p values (after Holm-Bonferroni correction) showing enrichment of phosphosites with a given property are denoted with an asterisk (*). ASA, accessible surface area.

^a ASA of a given protomer without binding partner.

^b Difference in ASA upon complex formation.

^c $\Delta\Delta\Delta G^{\text{Ala}}$ is the difference in binding energy upon Ala substitution. $\Delta\Delta\Delta G^{\text{P}}$ is the difference in binding energy upon attaching/removing phosphate groups to phosphorylation sites on interfaces. The p values for $\Delta\Delta\Delta G^{\text{P}}$ indicate whether the distribution is significantly shifted to positive values. See also Figure S1 and S2.

Thorn, 1998; Tuncbag et al., 2009). They are predominantly located on interaction interfaces, and their substitution by different amino acids (for example, Ala) causes large differences in binding energy (more than 1-2 kcal/mol), destabilizing the complex. The effect of such substitutions and therefore the contribution of a given site to the binding energy can be measured in terms of $\Delta\Delta\Delta G^{\text{Ala}}$ (see [Experimental Procedures](#)). We performed substitutions of Tyr/Thr/Ser residues in phosphoproteins from our test set by Ala (computational alanine scanning experiments) and calculated $\Delta\Delta\Delta G^{\text{Ala}}$ separately for phosphorylation and nonphosphorylation sites using the FoldX algorithm (see [Experimental Procedures](#)). Overall, the substitution of amino acids at both phosphorylation and non-phosphorylation sites destabilizes the complex, and the $\Delta\Delta\Delta G^{\text{Ala}}$ distributions are significantly shifted to positive values for all homo- and heterooligomeric complexes (p values = 2.2e-16 for both). We did not detect any Ala substitutions that would result in increased stability of the native complex by more than 2 kcal/mol (negative values of $\Delta\Delta\Delta G^{\text{Ala}}$ correspond to stabilizing substitutions). This implies that the interfaces are relatively well optimized, which is congruent with the previous studies (Brock et al., 2007).

Even though the majority of substitutions on interfaces do not change the binding energy very much, a significant fraction of them (10% for homooligomers and 13% for heterooligomers) contribute to a $\Delta\Delta\Delta G^{\text{Ala}}$ of more than +2 kcal/mol (destabilizing the complex); in other words, they form binding hotspots. We considered whether phosphorylation events tend to involve binding hotspots. We found that for heterooligomers, the $\Delta\Delta\Delta G^{\text{Ala}}$ values for amino acid substitutions at phosphorylation sites on binding interfaces are larger compared to other sites on interfaces (Wilcoxon rank-sum test, p value = 0.0003); namely, 7% of nonphosphorylation sites

and 13% of phosphorylation sites correspondingly contribute more than 2 kcal/mol to $\Delta\Delta\Delta G^{\text{Ala}}$ (20% of nonphosphorylation sites and 30% of phosphorylation sites have a $\Delta\Delta\Delta G^{\text{Ala}}$ of more than 1 kcal/mol). In general, the association between phosphosites and binding hotspots is statistically significant for the entire dataset, and for heterooligomers in particular (Fisher’s exact test, p value = 0.0006). This result does not hold true if only homooligomers are considered (Table 1; Figure S2).

As mentioned previously, the majority of protein complexes in PDB do not have actual phosphate groups present. Therefore, to further assess the energetic effect of phosphorylation, we attached the phosphate group to those Ser/Thr/Tyr sites on binding interfaces that are known to be phosphorylated and calculated the change of binding energy upon phosphorylation as $\Delta\Delta\Delta G^{\text{P}}$ (see [Experimental Procedures](#)). In the majority of cases, phosphorylation resulted in very moderate changes in the estimated binding energy of about +0.5-1.5 kcal/mol. Experimental studies on MAPK cascade scaffold protein showed that introducing phosphate increases the dissociation energy by about 1.5 kcal/mol (Serber and Ferrell, 2007; Strickfaden et al., 2007). Nevertheless, overall, the $\Delta\Delta\Delta G^{\text{P}}$ distribution was significantly shifted toward positive values (Figure 2; p value is 1.3e-08 for homooligomers and 1.3e-12 for heterooligomers). Namely, in 39% and 35% of the cases, the attachment of a phosphate group destabilized the complex for hetero- and homooligomers, respectively, by more than +2 kcal/mol. The phosphorylation of heterooligomers caused slightly higher destabilization compared to homooligomers. On the other hand, there were 8 and 64 cases where phosphorylation resulted in $\Delta\Delta\Delta G^{\text{P}}$ values of less than -2 and -1 kcal/mol, respectively, leading to complex stabilization. There were 12 complexes in our test set where the actual phosphate group was resolved on

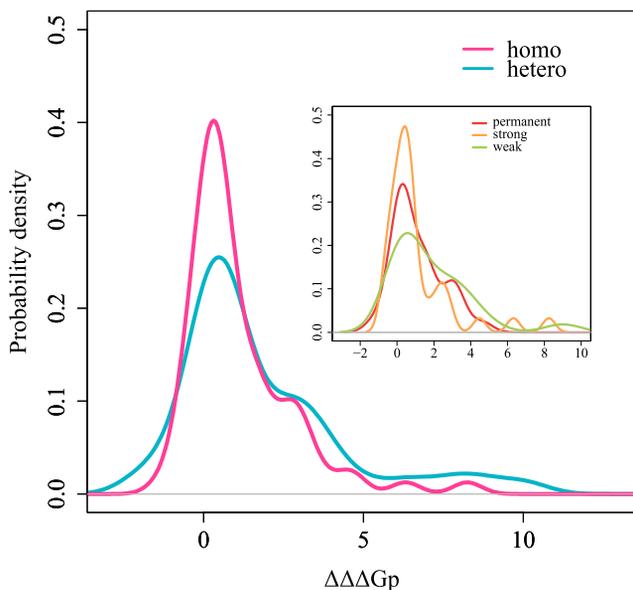


Figure 2. Probability Density Function of the Change in Binding Energy upon Phosphorylation for Interfacial Ser, Thr, and Tyr ($\Delta\Delta\Delta G^p$)

Curves for homooligomers ($n = 74$ phosphosites on interfaces) are shown in pink; curves for heterooligomers ($n = 104$) are shown in blue. Note that $\Delta\Delta\Delta G^p$ was calculated only for dimers due to the limitations of the program. The distribution is shifted toward positive values (p value = $2e-16$). Inset: permanent homooligomers are shown in red, strong transient homooligomers are shown in orange, and weak transient homooligomers are shown in green. The distributions are smoothed by the Gaussian kernel density estimation.

protein interfaces; in these cases, we removed the phosphate group and assessed the effect, and in most cases the $\Delta\Delta\Delta G^p$ was less than 2 kcal/mol.

Evolutionary Conservation of Phosphosites

The evolutionary conservation of phosphorylation sites has been a topic of several studies; it has been found that phosphoproteins are more conserved in evolution than nonphosphorylated ones (Boekhorst et al., 2008; Macek et al., 2008), whereas the conservation of phosphorylation sites is limited (Levy et al., 2010). One of the reasons for weak conservation of phosphorylation sites is that the majority of phosphorylation events might have occurred relatively recently in evolution, especially Tyr phosphorylation (Chen et al., 2010; Gnad et al., 2010; Sridhara et al., 2011). In an attempt to clarify this controversy, we mapped phosphorylation sites on multiple sequence alignments of manually curated Conserved Domain Database (CDD) families at the superfamily level and calculated their sequence conservation. Overall, 539 protein complexes from our dataset were mapped to 292 CDD families. First, we found in consensus with other studies (Boekhorst et al., 2008; Gnad et al., 2007; Gray and Kumar, 2011; Zanzoni et al., 2011) that phosphorylation sites are more conserved than the surface sites for heterooligomers (Wilcoxon rank-sum test, p value = 0.00001; Figure S3). Next, we went further and checked whether phosphorylation sites on interfaces are more conserved than other interface sites. Figure 3 shows the probability density plot of

sequence conservation calculated with respect to background conservation of the overall family for both phosphosites and all other Tyr/Thr/Ser sites on interfaces. This figure shows that heterooligomers, unlike homooligomers, have a small peak in the positive range of interface conservation values, which is consistent with previous studies (Choi et al., 2009). Moreover, the majority of nonphosphorylation sites on interfaces are less conserved than the family background (the mean value of the distribution is shifted toward negative values), which can be explained by the fact that protein core residues and active sites might be under stronger evolutionary pressure than Ser, Thr, and Tyr residues on interfaces.

When we look at the conservation of phosphorylation sites, it is evident that there are two almost equal populations of Ser/Thr/Tyr sites: those that are less conserved than the family background and those that are more conserved than the background. Overall, the conservation distribution for phosphosites is significantly shifted toward positive values compared to conservation of interfacial nonphosphosites for all complexes, and for heterooligomers in particular (p value = 0.018 for all; p value = 0.016 for heterooligomers). When calculated separately for homooligomers, this shift is not significant. Thus, we see that phosphosites are more conserved than nonphosphosites on interfaces in human complexes, implying that there is additional evolutionary pressure to conserve the phosphosites, which are important for binding events. This is also consistent with our previous observation that phosphosites in heterooligomers have a tendency to be located at the binding hot spots, and such hot spots are more evolutionarily conserved than the rest of the interface.

Functions of Phosphorylated Complexes

It has been reported that phosphorylated proteins have specific molecular functions in a cell (Wang et al., 2011). We analyzed our nonredundant set of homooligomers and heterooligomers, including phosphorylated and nonphosphorylated proteins (see Experimental Procedures), and studied their association with particular Gene Ontology (GO) protein functions. We found that heterooligomers with GO annotations “catalytic” (GO: 0003824), “hydrolase” (GO: 0016787), “transferase” (GO: 0016740), and “signal transducer” (GO: 0004871) activities have larger numbers of phosphorylation sites on interfaces compared to other proteins (Figure S4; Table S3).

It is also known that some pathways are differentially regulated by using reversible phosphorylation of their constituent proteins. In this respect, we performed an analysis of phosphorylated complexes participating in different biological pathways. The data on pathways were taken from the National Center for Biotechnology Information Biosystems database, which includes 5016 human specific pathways, mostly coming from KEGG and Reactome sources (Geer et al., 2010). We found that metabolic and hemostasis pathways (KEGG pathway ID: hsa01100; Reactome ID: REACT_604) were significantly enriched with phosphorylated homooligomeric complexes (p value = 0.002), whereas the “Hemostasis” (REACT_604), “Pathways in cancer” (hsa05200), “Cell Cycle, Mitotic” (REACT_152), and “Signaling in immune system” (REACT_6900) pathways were enriched with phosphorylated heterooligomeric complexes (p value = 0.00001; Table S3).

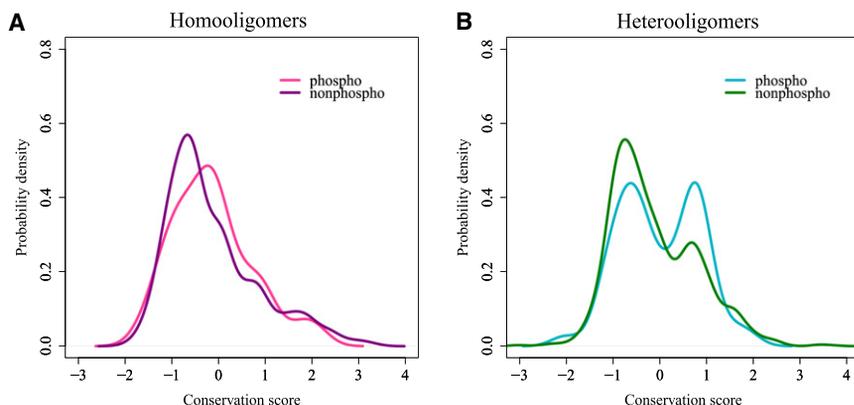


Figure 3. Probability Density Function of the Conservation Score Calculated for Phosphorylation Sites on Binding Interfaces

Zero conservation score corresponds to the same amount of evolutionary conservation as the mean conservation of the protein family.

(A) For homooligomers, conservation of phosphorylation sites ($n = 275$ phosphosites on interfaces) is shown in red and conservation of nonphosphorylation sites ($n = 2773$) is shown in purple.

(B) For heterooligomers, conservation of phosphorylation sites ($n = 521$) is shown in blue and conservation of nonphosphorylation sites ($n = 5559$) is shown in green. The conservation distribution for phosphosites is significantly shifted toward positive values compared to conservation of interfacial nonphosphosites for all complexes, and for heterooligomers in particular (p value = 0.018 for all and p value = 0.016 for heterooligomers). The distributions are smoothed by the Gaussian kernel density estimation. See also Figure S3.

Phosphorylation Mediates Complex Formation: Smad Proteins

Transforming growth factor- β signaling is controlled by receptor Ser/Thr kinases and the Smad protein family. In response to cytokine oligomerization, phosphorylation of Ser residues and subsequent activation of cytoplasmic Ser/Thr kinase occurs. Activated kinase then phosphorylates the C-terminal SSXS motif of specific tumor suppressors from the R-Smad (Smad1, Smad2) protein family. Once phosphorylated, the SSXS motif of Smad2 promotes the formation of a heterooligomer between R-Smad and Smad4, which in turn regulates gene expression. We compared a Smad1 protein from our test set (PDB accession 1khu) with a Smad2 protein (1khx) that has actual phosphate groups present in the crystal structure (except for the first Ser). Both structures have the SSXS motif located on the binding interface; moreover, these proteins are 80% identical and display extensive structural similarity (Figure 4A). We considered the effect of phosphorylation and dephosphorylation of Smad1 and Smad2 on trimer formation. We made a model of phosphorylated Smad1 and calculated the change in binding energy upon phosphorylation of Smad1 and also dephosphorylation of Smad2 (see Experimental Procedures). The model of phosphorylated state of the Smad1 is shown in Figure 4A; the different subunits are depicted in blue, green, and magenta, whereas the structure of the actual phosphorylated state of Smad2 protein is depicted in yellow. We showed that phosphorylation of all Ser, especially the third one in the SSXS motif, stabilized the complex of Smad1 (negative average $\Delta\Delta\Delta G^P$ values up to -1.5 kcal/mol; Table 2). At the same time, dephosphorylation of the second and especially third Ser destabilized the Smad2 complex by up to 2 kcal/mol. Removing the phosphate group of the first Ser slightly stabilized the complex.

The results of our computations are consistent with experimental results obtained for the Smad2 protein (Wu et al., 2001), and the effect of phosphorylation of the first Ser in the SSXS motif is still considered controversial (Abdollah et al., 1997). These experimental studies demonstrate that unphosphorylated Smad2 exists as a monomer, whereas phosphorylation of Smad2 promotes homotrimer formation through its MH2 domain (Wu et al., 2001). Interestingly, the trimer interface overlaps with the interface for the interaction between the

Smad2-MH2 domain and receptor Ser/Thr kinase domain, and as was shown previously, phosphorylation facilitates the dissociation of Smad2 from kinase (Wu et al., 2001). Therefore, this provides an example where phosphorylation mediates the complex formation and, through competitive binding, implements a negative control mechanism promoting the dissociation of the heterologomeric complex of Smad2 with the kinase domain.

DISCUSSION

We found that the vast majority of phosphocomplexes contain just a few phosphorylation sites, whereas for some proteins up to half of their sites (Ser, Thr, and Tyr sites) are potentially phosphorylated at some point, which is evident from the long tail of the probability distribution (Figure 1) for the fraction of phosphosites per protein. Several studies previously established that phosphosites may form clusters along specific regions of a protein sequence or on a protein surface (Schweiger and Linial, 2010; Yachie et al., 2009). Although the main reasons for these

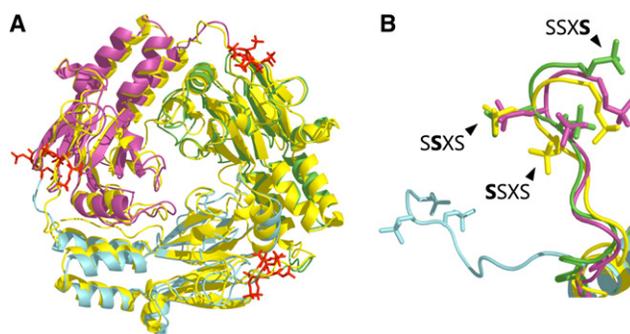


Figure 4. Phosphorylation in Smad1 and Smad2 Complexes

(A) Superposition of Smad2 structure (PDB ID: 1khx; yellow) and phosphorylated model of Smad1 generated by FoldX (based on 1khu; individual subunits are shown in magenta, green, and blue). Phosphorylated Ser462, Ser463, and Ser465 are colored in red.

(B) C-terminal loops of three subunits of phosphorylated Smad1 and Smad2. Colors of the subunits are the same as in (A), and phosphate groups are depicted in the same color as subunits.

Table 2. Effect of Phosphorylation/Dephosphorylation on Complex Formation of Smad Proteins

Protein	Site ^a	pSite	Dimer ^b			Average $\Delta\Delta\Delta G^c$
			AB	BC	AC	
Smad2 (1kx)	SSXS	S→pS	0.59	0.59	0.59	0.59
	SSXS	pS→S	0.88	0.88	0.88	0.88
	SSXS	pS→S	1.53	1.53	2.86	1.97
Smad1 (1khu)	SSXS	S→pS	-2.11	1.58	-0.22	-0.25
	SSXS	S→pS	-0.9	1.49	-1.74	-0.38
	SSXS	S→pS	-1.45	-1.87	-1.08	-1.47

Change in binding energy upon phosphorylation/dephosphorylation is calculated in terms of $\Delta\Delta\Delta G^P$ (negative and positive values correspond to stabilizing and destabilizing effects, respectively).

^a Phosphorylated/dephosphorylated positions in the SSXS motif are shown as boldface underscored characters.

^b Because FoldX can only handle dimeric complexes, Smad trimers were decomposed into the dimers AB, BC, and AC.

^c Average of all three pairs of chains.

findings remain largely unknown, it was observed that in some cases the groups of sites can be phosphorylated simultaneously and cooperatively, leading to certain advantages in terms of signal amplification and its strength modulation (Park et al., 2006). In our study, we showed that phosphosites have a tendency to be located on binding interfaces in protein complexes, and this trend depends on the type of complex. This might allow better understanding of the regulation of protein activity through phosphorylation within the framework of protein binding.

There are several reasons for such coupling between phosphorylation and binding. Phosphorylation may modulate the strength of interactions, bringing about changes in binding energy that may trigger the transitions between different conformer and oligomeric states. For the majority of proteins in our dataset, the phosphorylation did not change the binding affinity significantly, which is consistent with several experimental studies pointing to the modest effect of phosphorylation on stability and protein conformation (Murray et al., 1998; Serber and Ferrell, 2007; Strickfaden et al., 2007). At the same time, in one-third of our complexes the attachment of a phosphate group to interfacial Ser/Thr/Tyr sites, which are expected to be phosphorylated, caused a relatively large change in estimated binding energy. This in turn could lead to conformational changes or preclude undesired interactions due to steric constraints. Moreover, phosphorylation sites on interfaces significantly overlapped with the binding hot spots in heterooligomeric complexes, and phosphorylation at binding hotspots could potentially disrupt the complex formation. In addition, we showed that phospho Ser/Thr/Tyr on interfaces were more conserved than nonphosphorylated Ser/Thr/Tyr on interfaces. It should be mentioned that regulatory mechanisms of phosphorylation are quite diverse, and in some cases, phosphorylation might destabilize the complex and lead to protein activation or inactivation, whereas in others it may mediate complex formation and through competitive binding provide a negative control mechanism (as was shown in the Smad example). In our study, the phosphate group was attached to only one site at a time,

and since there can be several phosphosites per protein (on average there are about two phosphosites per protein in the set), we expect a greater effect if multiple sites are phosphorylated simultaneously.

Phosphorylation might not affect significantly complex stability, but rather provide diversity in recognition patterns and offer recognition sites for binding of certain domains and motifs (e.g., pTyr-binding by the SH2 domain, pSer/pThr binding by the MH2 and FHA domains), thereby modulating binding selectivity. Indeed, the reversibility of phosphorylation events allows decoupling of the binding specificity and affinity, thereby mediating specific binding even between proteins within transient and not very stable complexes. At the same time, phosphorylation of multiple sites on interfaces may amplify this signal and provide enhanced binding selectivity. Indeed, such specific and reversible signaling at the residue level is a good indicator that a previous stage in cellular signaling networks has been completed successfully. Many cellular control mechanisms operate at the level of protein-protein interactions, and main signaling pathways involve dense networks of protein-protein interactions and phosphorylation events. Moreover, signaling pathways are quite often disrupted in cancer, and it was recently shown that somatic cancer mutations are enriched with those that cause gain or loss of phosphorylation sites (Radivojac et al., 2008). Similarly, our study showed that the signaling pathways “Hemostasis,” “Pathways in cancer,” “Cell Cycle, Mitotic,” and “Signaling in immune system” are enriched with phosphorylated heterooligomeric complexes.

Interestingly, we found that metabolic and hemostasis pathways are also enriched with phosphorylated homooligomeric complexes, and phosphosites in weak transient homooligomers are considerably involved in binding. Previously, we manually compiled a set of experiments that furnish evidence that phosphorylation at or near the homooligomer interface shifted the equilibrium between different oligomeric states with different protein activities (Hashimoto et al., 2011). According to the classical model by Goldbeter and Koshland (1981), posttranslational modifications may allow large activity changes with only moderate concentration changes to provide sensitive response to external stimuli. To supplement this model, our analysis offers additional new evidence for how reversible phosphorylation events may modulate reversible transitions between different discrete conformations or oligomeric states in homooligomeric and heterooligomeric complexes and might represent an important mechanism for regulation of protein activity.

EXPERIMENTAL PROCEDURES

Data Set of Phosphorylation Sites

The data on phosphorylation sites in human proteins is derived from the PhosphoSitePlus (Hornbeck et al., 2004), Phospho.ELM (Dinkel et al., 2011), and PHOSIDA (Gnad et al., 2007) databases. Most phosphorylation sites in these databases are identified by high-throughput (HTP) methods that might contain significant experimental errors (Lin et al., 2010). Therefore, we used the GPS 2.1 program (Xue et al., 2008) to verify HTP phosphosites. GPS predicts phosphorylation sites from protein sequence based on sequence patterns using decision trees. In our study, we employed the most conservative thresholds reported by GPS with the estimated false-positive rates being 2% and 4% for the Ser/Thr and Tyr sites, respectively. The sites identified by low-throughput methods (indicated as “PUBMED_LTP” in PhosphoSitePlus and “LTP” in Phospho.ELM) and HTP sites verified by GPS were then used

for our analysis. Proteins with reliable phosphorylation sites were linked to their structures via Uniprot (Magrane and Consortium, 2011), and the phosphorylation sites were mapped onto PDB structures using the Muscle alignment algorithm (Edgar, 2004). A protein list with phosphosites and all results is available at ftp://ftp.ncbi.nih.gov/pub/mmdb/phospho/phosphorylation_on_complexes.xls.

Data Set of Phosphorylated Protein Complexes

We started our analysis with the whole set of PDB structures (Berman et al., 2000) and retrieved all structures containing more than one protein chain. The oligomeric states and binding interfaces were defined using the Protein Interfaces, Surfaces and Assemblies (PISA) algorithm (Krissinel and Henrick, 2007). PISA is considered a standard, state-of-the-art method that detects biological macromolecular assemblies in PDB with 80%-90% accuracy. We regard a complex as stable if PISA reports a unique oligomeric state for a given structure. A complex is considered homooligomeric if sequence identities between all chain pairs in the complex are more than 90% identity, otherwise it is defined as heterooligomeric. Phosphorylation sites were mapped to protein complexes, and then to compile a nonredundant set, similar proteins (with BLAST, p value $< 10e-07$) were removed. Finally, we obtained a nonredundant set of 382 homooligomers and 551 heterooligomers with 1983 phosphorylation sites altogether. Homooligomers were further divided into three categories similar to the classification introduced recently by Perkins et al. (2010). According to their ΔG of dissociation calculated by PISA, we distinguished the following categories: weak transient ($\Delta G_{diss} \sim 0$, coexistence of different oligomeric states), strong transient ($0 \leq \Delta G_{diss} \leq 20$ kcal/mol), and permanent ($\Delta G_{diss} > 20$ kcal/mol) homooligomers. For reference, for dimers in equilibrium, the concentrations of dimers and monomers are equal at $\Delta G_{diss} \sim 4$ kcal/mol (Krissinel and Henrick, 2007).

Calculation of Binding Energies

The change in the standard free energy upon complex dissociation may be calculated as follows:

$$\Delta G_{diss}^0 = -RT \log K_D = -RT \log \frac{\pi_i^n [M_i]}{[C]} \quad (1)$$

where K_D is the dissociation constant and $[M]$ and $[C]$ are equilibrium standard-state concentrations of complex C and monomers M . Dissociation energy was calculated with PISA and was used to assess the complex stability. Since amino acid substitution and phosphate attachment/removal can affect the stability of both monomers and complexes, we also estimated the binding energy with the rigid body approach using the same atomic coordinates for the monomers as in the complexes:

$$\Delta \Delta G_{bind} = \Delta G_C - \sum_i^n \Delta G_{M_i} \quad (2)$$

where ΔG_C and ΔG_{M_i} are stabilities (unfolding free energies) of the complex and monomers, respectively.

The computational alanine scanning and attachment/removal of phosphate groups was performed by the FoldX program (Guerois et al., 2002; Sanchez et al., 2008) using the “complex_alascan” and “PositionScan” options, respectively. The FoldX program calculates the stability of protein complexes using an empirical force field. As was shown previously, FoldX is among the best three methods used to estimate the effect of mutations on protein stability. It reaches 0.64 sensitivity and 0.43 specificity (Khan and Vihinen, 2010) of prediction and reports a correlation coefficient between experimental and computed $\Delta \Delta \Delta G$ values in the range of 0.5-0.8 (Guerois et al., 2002; Potapov et al., 2009), with the SD of computed $\Delta \Delta G$ values being 0.8 kcal/mol (Guerois et al., 2002). In “complex_alascan” mode, FoldX replaces the residue on the interface by alanine, optimizes their side chain conformations, and calculates the difference in binding energies between the original and substituted complexes ($\Delta \Delta \Delta G^{al}$). Similarly, in “PositionScan” mode, FoldX attaches/removes a phosphate group to/from Ser/Thr/Tyr, optimizes the side chain conformations, and calculates the difference in binding energies between the original and phosphorylated complexes ($\Delta \Delta \Delta G^P$). Positive and negative values of $\Delta \Delta \Delta G$ correspond to destabilizing and stabilizing effects, respectively. Note that $\Delta \Delta \Delta G^P$ was calculated only for dimers due to limitations of the program.

Analysis of Evolutionary Conservation of Phosphorylation Sites

To examine the evolutionary conservation of phosphorylation sites, we searched protein sequences from our data set using RPS-BLAST (Marchler-Bauer et al., 2002) and the CDD (Marchler-Bauer et al., 2009) and then embedded the protein sequences in the CDD multiple sequence alignments. The conservation score was calculated using the al2co program (Pei and Grishin, 2001) with default parameters. It represented the entropy-based measure calculated from sequence weighted observed amino acid frequencies. The score was normalized by subtracting the mean and dividing by the SD of the score distribution for the whole alignment. Therefore, the conservation score of a given site can be negative if the site is less conserved than the average conservation background of protein family, and vice versa.

Annotating Protein Function and Functional Pathways

We used GO (Ashburner et al., 2000) for the annotation of the protein function. The “molecular function” terms of each protein were obtained from Gene Ontology Annotation database (Barrell et al., 2009). For pathway analysis, we used the NCBI BioSystems Database (Geer et al., 2010) and Flink web service (<http://www.ncbi.nlm.nih.gov/Structure/flink/flink.cgi>) to map proteins to biological pathways. The content of the Biosystems Database comes from several pathway databases: KEGG (Kanehisa et al., 2010), BioCyc (Caspi et al., 2010), PID (Schaefer et al., 2009), and Reactome (Croft et al., 2011). Only human-specific pathways were considered for our analysis. In addition to the set of phosphorylated protein complexes described above, nonredundant sets of homooligomers and heterooligomers, including phosphorylated and nonphosphorylated proteins, were compiled to estimate whether phosphorylated proteins were enriched in specific function or pathways. All human complexes were taken from PDB and validated with PISA, and similar proteins (with BLAST p value $< 10e-07$) were then removed as described previously. The final data set contained 248 phosphorylated and 451 nonphosphorylated homooligomers and 253 phosphorylated and 401 nonphosphorylated heterooligomers, respectively.

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures and three tables and can be found with this article online at <doi:10.1016/j.str.2011.09.021>.

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