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Review Article (Invited)

Charge block-driven liquid-liquid phase separation: A mechanism of how phosphorylation regulates phase behavior of disordered proteins

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Phosphorylation regulates protein function by modulating stereospecific interactions between protein-protein or enzyme-ligand. On the other hand, many bioinformatics studies have demonstrated that phosphorylation preferably occurs in intrinsically disordered regions (IDRs), which do not have any secondary and tertiary structures. Although studies have demonstrated that phosphorylation changes the phase behavior of IDRs, the mechanism, which is distinct from the "stereospecific" effect, had not been elucidated. Here, we describe how phosphorylation in IDRs regulates the protein function by modulating phase behavior. Mitotic phosphorylation in the IDRs of Ki-67 and NPM1 promotes or suppresses liquid-liquid phase separation, respectively, by altering the "charge blockiness" along the polypeptide chain. The phosphorylation-mediated regulation of liquid-liquid phase separation by enhancing or suppressing "charge blockiness," rather than by modulating stereospecific interactions, may provide one of the general mechanisms of protein regulation by posttranslational modifications and the role of multiple phosphorylations.

Key words: biological condensates, intrinsically-disordered region, post-translational modifications, charge block

Significance

The new finding that phosphorylation modulates the behavior and function of the target protein by changing the charge block-driven liquid-liquid phase separation propensity can answer the following biological questions regarding phosphorylation and other posttranslational modifications. I) Why do phosphorylation and other posttranslational modifications preferably occur in intrinsically disordered regions? II) Why does phosphorylation occur in close neighbors along the polypeptide chain? III) Why is the consensus of the flanking sequence of the phosphosite low? Together with the conventional "stereospecific" mechanism, it contributes to our understanding of a global picture of the phosphoproteome and how it regulates various intracellular reactions and events.

Introduction

Posttranslational modifications (PTMs) of cellular proteins play essential roles in the regulation of protein function and dynamics, signal transduction, and cell cycle progression. Comprehensive analyses of PTMs using mass spectrometry and other approaches have identified more than 40,000 phosphorylated residues in more than 10,000 proteins in the human proteome, which are regulated by more than 520 kinases and 150 phosphatases [1,2]. Therefore, elucidating the whole

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picture and dynamics of the phosphoproteome is necessary to understand cellular mechanisms.

The three-dimensional structure of a polypeptide produces a “stereospecificity” in protein-protein, protein-small molecule, and enzyme-ligand interactions, which determines the functional specificity of the protein and enzyme. The stereospecificity, which is often analogized to a relation between a “key” and a “keyhole”, is one of the most important elements in understanding the protein function and has been revealed by crystallography, nuclear magnetic resonance spectroscopy, and single-particle analysis using electron microscopy. This mechanism also plays a pivotal role in the regulation of protein function via PTMs. For example, phosphorylation covalently attaches a phosphate group to the hydroxyl group of a specific serine, threonine, or tyrosine residue, and locally adds a negatively charged small moiety to the bulk body of the protein molecule. Nevertheless, it drastically changes the molecular interaction directly when it occurs on the interaction surface (orthosteric) or indirectly when it induces a conformational change in the protein (allosteric). The conformational changes caused by the phosphorylation of the carboxyl-terminal domain of the actin-binding protein dematin and the change in the cis-trans isomerization energy barrier of proline residues are good examples of the allosteric effects of phosphorylation [3,4]. Hence, it is widely accepted that phosphorylation regulates protein function by altering stereospecific interactions. However, it remains unclear whether this mechanism can explain all the 40,000 phosphosites. To date, only a few thousand phosphosites have been characterized. This review article is an extended version of the Japanese article [5].

Intrinsically Disordered Regions (IDRs) are Major Targets of PTMs

Recent advances in proteomic approaches have revealed that phosphorylation preferentially occurs in IDRs, which do not have secondary or tertiary structures [6–8]. Our quantitative phosphoproteomic analysis using tandem-mass-tag mass spectrometry identified more than 10,000 sites that are phosphorylated upon entry into mitosis, more than half of which reside in long IDRs (> 30 residues) [7]. Given that serine/threonine residues in IDRs constitute approximately 20% of the total, this is a notably high frequency. This tendency can be found not only in phosphorylation, but also in other PTMs, such as methylation and acetylation [8]. These findings obviously challenge and contradict our conventional understanding of “stereospecific” regulation of protein function by phosphorylation. Although it is possible that phosphorylation induces a conformational change in IDRs, such cases have only been reported for very short peptides [9]. Therefore, elucidating the structural and functional roles of phosphorylation in IDRs is necessary to understand the entire phosphoproteome.

Phosphorylation Affects the Phase Behavior of IDRs

Since 2013, many biologists and biophysicists have begun to shed light on new characteristics of IDRs. They found that intracellular membrane-less organelles (MLOs), such as nucleoli and stress granules, are formed via liquid-liquid phase separation (LLPS), and that IDRs play a major role in the formation of MLOs [10,11]. A solution system containing soluble polymers is phase-separated into two phases: one with a high polymer concentration (condensed phase) and the other with a low polymer concentration (diluted phase) when the polymer concentration in the system increases or the interaction between polymers increases. In biological systems, weak multivalent interactions between IDRs and nucleic acids often induce LLPS. As the driving forces of such weak interactions are electrostatic, cation-π, and π-π interactions, the phase behavior of IDRs largely depends on the amino acid composition and sequence. As phosphorylation adds negative charges to the polypeptide chain, it can be speculated that phosphorylation affects the phase behavior of the IDR. Several studies have demonstrated that phosphorylation regulates the assembly/disassembly of MLOs [12–15]. Although LLPS-prone IDRs have a strongly biased amino acid composition, the sequence dependency of LLPS is not fully understood. Therefore, the mechanism by which phosphate groups added to the IDR modulate the phase behavior is largely unknown.

Modulating the Charge Block Pattern by Phosphorylation

To address this question, our research group focused on mitotic phosphorylation, which is catalyzed by mitotic kinases, such as CDK1/cyclin B, and plays pivotal roles in the morphological changes of intracellular organelles, including MLOs (such as nuclear envelope breakdown, chromosome condensation, and nucleolar disassembly) [16]. NPM1 is an IDR-rich nucleolar protein that plays a major role in the liquid-like properties of the nucleolus. Ki-67 is an IDR-rich protein that is mainly localized at the periphery of the nucleolus (perinucleolar region). Upon entry into mitosis, both proteins are heavily phosphorylated, NPM1 starts to dissolve in the cytoplasm, and Ki-67 starts to localize to the mitotic chromosome periphery, which is supposed to have liquid-like properties. Using quantitative phosphoproteomics, we identified 15 residues in NPM1 and more than 100 residues in Ki-67 that are phosphorylated during mitotic entry (Fig. 1a) [7]. *In vitro* LLPS assays using purified recombinant proteins have shown that phosphorylation by CDK1/cyclin B suppresses and

promotes the LLPS of NPM1 and Ki-67, respectively [16]. To elucidate this mechanism, we examined the charge distribution along the IDRs and identified the regions with segregated charges (positive and negative charge blocks). Interestingly, 11 mitotic phosphosites in the NPM1 IDR existed within two positively charged blocks. Assuming that a phosphate group adds a negative charge of -2, the two positive charge blocks were diminished by 11 phosphorylations (Fig. 1b). In contrast, a single repeat unit of Ki-67 (12th repeat [R12]) has a positively charged block in the amino-terminal half, and most of the nine mitotic phosphorylations occur in the carboxyl-terminal half, which produces a negatively charged block (Fig. 1b). Therefore, mitotic phosphorylation has opposite effects on the charge block patterns of NPM1 and Ki-67; it diminishes the charge blocks of NPM1 and enhances those of Ki-67.

In soft matter physics, the relation between the charge distribution and the phase behavior of polyampholytes has been studied. In these studies, the LLPS propensity of the polyampholyte chains consisted of an equal number of positively and negatively charged monomers (the total charge was 0) with different monomer sequences were compared. Notably, they found a positive correlation between the charge block size and the LLPS propensity [17–20]; a polymer with large charge blocks showed stronger LLPS than that with a random distribution. Although the mechanism of this "charge block-driven LLPS" has not yet been fully elucidated, it might be due to the counteracting effect of the coexistence of attractive and repulsive forces in a random polyampholyte, which work between the monomers with opposite and like charges, respectively. When the mechanism of charge block-driven LLPS is applied to the phosphorylation of polypeptides, it can be speculated that phosphorylation, which reduces the charge blockiness, diminishes the LLPS propensity (as is the case for NPM1), whereas that which increases the charge blockiness enhances LLPS (as is the case for Ki-67) (Fig. 1c).

To experimentally prove this, we generated two mutants of NPM1 and Ki-67: (1) a phosphomimetic mutant, in which all mitotic phosphosites were replaced with glutamic acid, and (2) a charge block-mimetic mutant, in which non-phosphorylatable residues were replaced with glutamic acid to mimic the charge distribution pattern of the phosphorylated form. *In vitro* LLPS assays using these mutants showed that the LLPS propensity of both mutants (1 and 2) decreased in Ki-67 and increased in NPM1 compared with that of the wild type (Fig. 2a). To exclude the possibility of an increase in the net negative charges by the mutation, we constructed a series of mutants with various charge blocks and total charges, and examined both parameters. The results showed that the LLPS propensity was strongly correlated with charge blockiness and not with the net charge (Fig. 2b). Furthermore, we examined the behavior of these mutants in cultured cells and found that the formation of MLOs was tightly correlated with the charge blockiness of NPM1 and Ki-67; Ki-67 mutants formed the chromosomal periphery in mitotic cells (Fig. 2c), and NPM1 mutants leaked out of the nucleolus in interphase cells (Fig. 2d). These results demonstrate a strong correlation between the charge block pattern of IDR and LLPS propensity. In addition, expression of Ki-67 mutants in Ki-67 knockout cells rescued this phenotype. These results demonstrated that mitotic phosphorylation regulates protein function by changing the charge block pattern of IDR and its LLPS propensity.

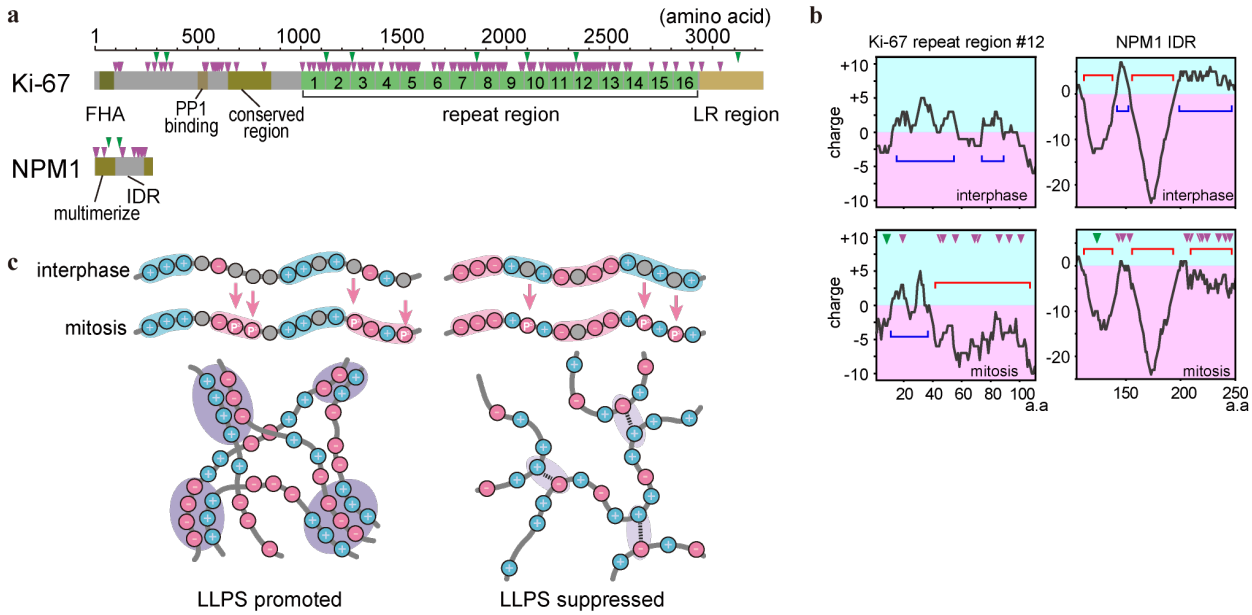

Figure 1 (a) Domain structures and phosphorylation sites of Ki-67 and NPM1. Mitotic phosphosites and dephosphosites are shown in purple and green triangles, respectively. (b) Charge plots of the interphase (top) and mitotic (bottom) forms of Ki-67 and NPM1. Positive and negative charge blocks are indicated with blue and red brackets, respectively. (c) Phosphorylations promote (left) and suppress (right) LLPS. Adopted from ref. [16] with modifications.

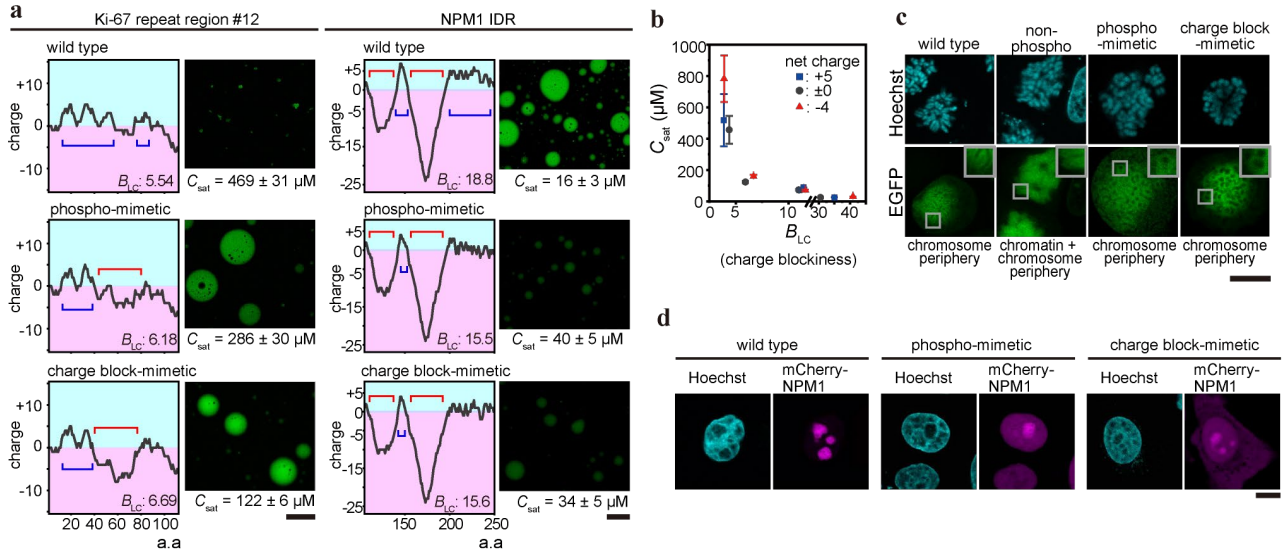

Figure 2 (a) Charge plots of Ki-67 (left) and NPM1 (right) and fluorescence images of droplets formed *in vitro*. B_{LC} indicates the segregation of charges (charge blockiness), and C_{sat} represents the critical concentration of phase separation. (b) The relation between C_{sat} and B_{LC} , obtained from various mutants of Ki-67. (c) Mitotic localization of EGFP-fused Ki-67 mutants in HeLa cells. The wild-type, phosphomimetic, and charge block-mimetic forms localized in chromosomes periphery (outside Hoechst signal), whereas non-phosphorylatable form localized inside the chromosomes (within Hoechst signal). (d) Intracellular localization of wild-type and mutant mCherry-NPM1 in interphase HeLa cells. Bars: 50 μm (a), 10 μm (c, d). Adopted from ref. [16] with modifications.

Summary and Prospects of Charge Block type LLPS Regulation

Charge block-driven LLPS and its phosphorylation regulation can be summarized as follows: (1) The change in the charge block pattern due to multiple phosphorylations rather than residue-specific phosphorylation is important. (2) In a single-component polymer solution, the LLPS was enhanced when both positive and negative blocks were present in the IDR. If only one of the blocks is present or if there is no clear block, LLPS is unlikely to occur. (3) The charged block is often several tens of amino acids in size. In addition, a recent study demonstrated that charge block patterns define the spatial distribution of the condensate and spatially regulate the function; positive and negative transcriptional regulators, which carry different charge block patterns, co-existed or excluded RNA pol II in the condensate and regulate transcriptional activity at a certain gene locus [21]. This suggests that charge block-driven LLPS may play a role in defining the spatial distribution of biological condensate within a cell.

In the conventional “stereospecific” regulation of protein function by phosphorylation, the position and the residue of the phosphosite are critical, and, therefore, many important phosphosites are evolutionarily conserved. In contrast, the macroscopic charge property of the IDR plays a major role in charge block-driven LLPS. Generally, the amino acid sequences of IDRs are less conserved than those of the structured domains. The same tendency was observed in the phosphosites in the IDR; only six out of 11 phosphosites in human NPM1 were conserved in *Xenopus laevis* (Fig. 3a). In contrast, the charge block patterns of these species were highly similar (Fig. 3b), indicating the importance of the macroscopic properties of charge distribution. In addition, charge block-driven LLPS may provide an answer to the question of why multiple phosphorylation occurs in IDRs and why they occur in a close neighbor [6,22,23]. Since a single phosphate group has little effect on the charge block properties, it can be speculated that multiple phosphorylations need to be segregated to change the block properties. Indeed, the frequency of clustered phosphosites (i.e. two phosphosites exist within 10 amino acids residues) is 3 times higher in IDRs than in non-IDRs [7].

Analysis of the charge distribution of IDRs and the position of mitotic phosphosites in the human proteome revealed that the mitotic behaviors of many cellular proteins other than NPM1 and Ki-67 are also regulated by charge block-driven LLPS [16]. Since the intracellular environment contains several different species of proteins, nucleic acids, and small molecules, it is necessary to describe and understand the behavior of a system that contains multiple components. Further studies are required to address this issue.

As PTMs other than phosphorylation also regulate LLPS [24,25], it is likely that the charge block-driven LLPS also works with other PTMs that affect the charge properties of the IDR, such as acetylation and methylation. Acetylation

occurs on the lysine and arginine side chains and removes positive charges. Methylation, which also occurs at lysine and arginine residues, does not eliminate the positive charge, but is thought to mask the positive charge of the amino group with methyl group(s). As both PTMs are known to segregate in IDRs [8], they are likely to reduce positively charged blocks and LLPS propensity. This possibility should be investigated in future studies. A technique using nuclear magnetic resonance spectroscopy to characterize the surface charge of IDRs could be helpful for this [26].

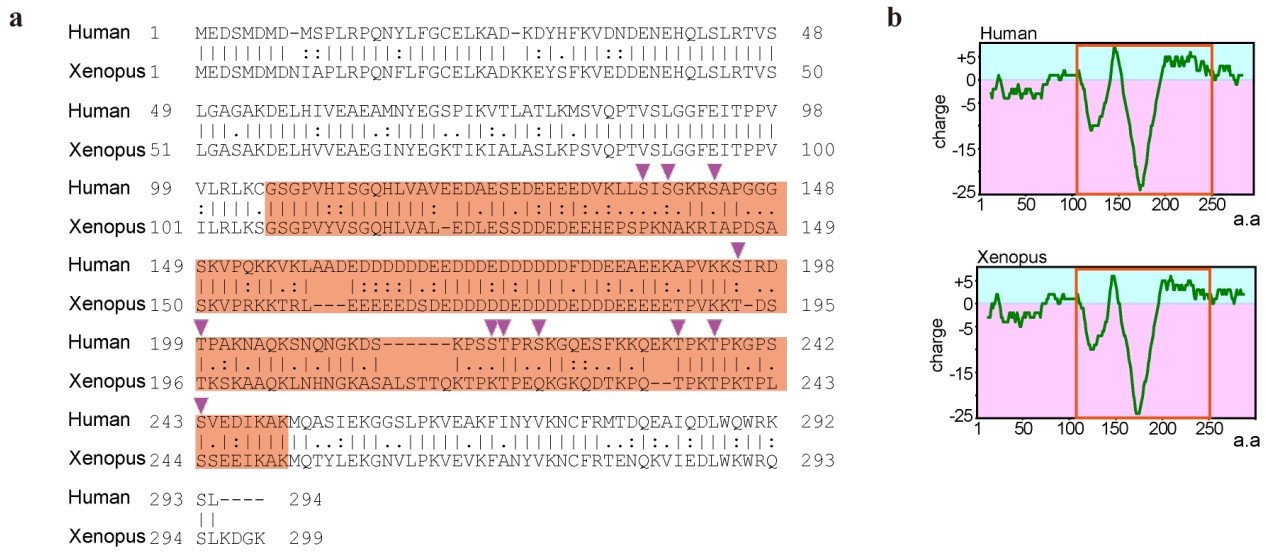

Figure 3 (a) Amino acid sequence alignment of human and *Xenopus laevis* NPM1. Human IDRs are shaded in orange, and human mitotic phosphosites are indicated with triangles. (b) Charge plots of human and *Xenopus laevis* NPM1 (window size: 25 amino acids). IDRs are indicated with a red box.

Conclusion

Phosphorylation regulates protein function by affecting the conventional stereospecific interactions with substrates or partner proteins. However, as discussed in this review, recent studies have demonstrated that phosphorylation preferentially occurs in IDRs and modulates the phase behavior of the protein. Mitotic phosphorylation of the nucleolar proteins Ki-67 and NPM1 promotes or inhibits LLPS by enhancing or reducing charge blockiness, respectively. Despite the low amino acid sequence conservation of the IDRs, the charge block pattern appears to be highly conserved among different species. The phosphorylation-mediated regulation of protein function by enhancing or reducing charge blockiness, rather than by modulating stereo- and site-specific interactions, provides a new mechanism of protein regulation by PTMs.

Conflict of Interest

The authors declare no competing or financial interests.

Author Contributions

HY and SHY conceptualized the topic of this article. HS, HY, and SHY wrote the manuscript.

Data Availability

All data used in this review originated from the corresponding articles.

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