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Current Opinion in Structural Biology

Liquid–liquid phase separation in cellular signaling systems

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Liquid–liquid demixing or phase separation of protein with RNA is now recognized to be a key part of the mechanism for assembly of ribonucleoprotein granules. Cellular signaling also appears to employ phase separation as a mechanism for amplification or control of signal transduction both within the cytoplasm and at the membrane. The concept of receptor clustering, identified more than 3 decades ago, is now being examined through the lens of phase separation leading to new insights. Intrinsically disordered proteins or regions are central to these processes owing to their flexibility and accessibility for dynamic protein–protein interactions and post-translational modifications. We review some recent examples, examine the mechanisms driving phase separation and delineate the implications for signal transduction systems.

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Current Opinion in Structural Biology 2016, **41**:180–186

This review comes from a themed issue on **Multi-protein assemblies in signaling**

Edited by **Arun K Shukla** and **Shoshana J Wodak**

For a complete overview see the Issue and the Editorial

Available online 20th August 2016

http://dx.doi.org/10.1016/j.sbi.2016.08.001

Introduction

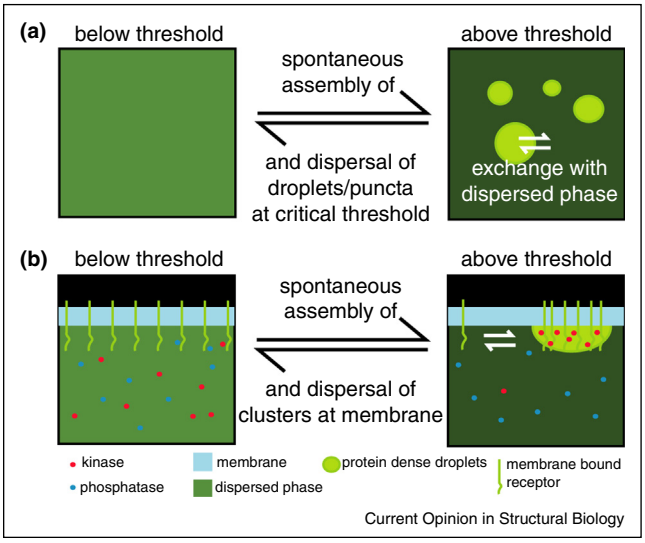
Assembly of multi-component protein complexes is accepted as an important process for signal transduction [1–3]. More recently some signaling complexes have been demonstrated to be highly dynamic with the properties of a separate protein-rich liquid phase. Liquid–liquid demixing or phase separation of proteins has been identified as a mechanism for formation of membraneless compartments, especially ribonucleoprotein granules [4,5••,6,7,8•], but is also emerging as an important concept in signaling. Examples include phase separation of Dishevelled (Dvl) in the Wnt signaling pathway [9–11], which is important in development, and phase separation of nephrin/Nck/N-WASP proteins[12••,13••], which function in the assembly of actin filaments [14,15]. Phase separation has been demonstrated to rely on multivalent protein interactions [12••,13••] and often involves intrinsically disordered proteins (IDPs) or proteins that contain intrinsically disordered regions (IDRs). The frequent occurrence of multiple modular domains in signaling proteins [2] and IDRs in signaling hubs [16–20] makes it likely that these phase-separation mechanisms are common in signaling. Three-dimensional membraneless compartments in the cytoplasm are also called membraneless organelles, puncta or droplets, alluding to their characteristics. Membraneless refers to these droplets not being membrane delimited or contained. However, the phase-separated proteins can be linked to a membrane through a membrane-bound protein component, leading to a pseudo two-dimensional phase separation at the membrane surface. Such two-dimensional phase separation at the membrane is closely tied to and may be the driving force behind receptor clustering. Phase separation allows for the creation of a microenvironment with emergent properties that may be useful for sequestering substrates or enzymes or creating specialized reaction environments [5••,21•,22,23]. Phase separation also provides an added layer of control in cell signaling and a mechanism for switch-like behavior and for integrating multiple inputs [11,12••].

Dvl: an early example of phase separation in signal transduction

Dvl is an intracellular protein that transmits Wnt signals to cytoplasmic targets in response to binding of extracellular Wnt by the Frizzled receptor [10,24]. Dvl or Dvl2, a mammalian form of Dvl, either overexpressed or endogenous, can form cellular puncta [25–27]. These puncta were initially assumed to represent Dvl protein associated with cytoplasmic membrane-bound vesicles. However, extensive testing of this hypothesis demonstrated that Dvl2 puncta were not associated with a membrane [10,28], but were in fact composed almost entirely of Dvl2-GFP fusion protein (estimated at 500–2500 mg/ml).

Puncta, which form when a critical threshold protein concentration is reached, behave as a separate liquid phase within the cytoplasm. Fluorescence recovery after photobleaching (FRAP) experiments indicated a rapid exchange of Dvl2 protein between the protein-rich puncta and the more diffuse Dvl2 protein in the cytoplasm (Figure 1a), providing evidence of the highly dynamic nature of the puncta. Studies on an increasing number of phase-separating proteins indicates that their viscosity in the phase-separated state can range from 10^3

Figure 1



(a) At the critical threshold, phase-separating proteins in the cytoplasm (green) spontaneously self-associate to form a separate protein-rich liquid phase (bright green circles), leading to depletion of the phase-separating protein in the cytoplasm (darker green). Proteins within the phase-separated state diffuse within the droplets, although the viscosity within the droplets is generally higher than the viscosity of the cytoplasm. There is also exchange of the protein between the protein-rich droplets and the much lower concentration of protein in the cytoplasm. Droplets disperse when the conditions drop below the critical threshold (change of protein or binding partner concentration or solvent condition). **(b)** When a protein involved in phase separation is membrane-bound, phase separation occurs at the membrane leading to clustering. The phase-separated droplets can enrich certain factors and exclude others. In this example, kinases that phosphorylate the receptor are enriched, whereas phosphatases are excluded.

to 10^6 times the viscosity of water (1–10^3 Pa s) [22]. The size of the puncta varied between 0.2 and 2 μm depending on Dvl2 concentrations. Consistent with a liquid state, smaller puncta could be observed fusing to form larger particles.

More recent *in vitro* studies support the idea that liquid–liquid protein phase separation creates the matrix of cellular puncta and other membraneless bodies. A study of Ddx4 [5••] showed that the formation of protein-rich phase-separated droplets in live cells by a Ddx4-GFP fusion could be reproduced *in vitro*, demonstrating that these membraneless compartments can assemble spontaneously from solutions of highly purified protein. Purified hnRNPA1 and LAF-1 have also been demonstrated to form protein-rich phase-separated droplets *in vitro* [8,29••]. These *in vitro* studies suggest that protein concentration thresholds for phase separation are in the 10^{-4} to 10^{-6} M range [5••,8•,12••]. The difference in concentration between the protein dense and depleted phases is approximately 100 fold ([12••] and JP Brady *et al.* personal communication). FRAP studies on multiple systems indicate that recovery of fluorescence following whole droplet bleaching occurs on a timescale of seconds to minutes, pointing to rapid exchange between the protein dense and depleted phase [8•,10,13••]. Thus, phase separation by Dvl exemplifies a pattern that is observed for other phase-separating proteins and likely drives formation of cellular bodies and puncta.

The signaling activity of Dvl mutants is strongly correlated with the ability of these mutants to form puncta [10,28,30]. In fact, overexpression of Dvl, which promotes puncta formation, can result in Wnt-independent signaling (see references in [9]). While the possibility that signaling-active Dvl polymers are smaller than the puncta observed in cells cannot be fully discounted [9], the evidence suggests that Dvl puncta mediate Wnt signaling. Notably, the spontaneous and rapid nature of initial puncta formation introduces switch-like behavior into Dvl signals resulting in rapid shifting between the on and off states [11].

The role of multivalency, affinity and concentration in phase separation

What properties enable some proteins to form separate liquid phases? The first requirement is likely an ability to engage in multiple interactions. Mechanistic studies employing pairs of synthetic constructs, with one member of the pair containing a variable number of SH3 domains and the other member a variable number of proline-rich motifs (PRM), support a requirement for multivalent interactions [12••,13••]. The ability of these protein pairs to phase separate was shown to depend strongly on the number of SH3 domains and PRM repeats in the construct pairs. High-affinity monovalent ligands could block phase separation by effectively reducing the degree of valency. When five SH3 domains are present in one partner and 5 PRM repeats are present in the second partner, phase separation occurs with both proteins in the low μm range. By contrast, with three SH3 domains and three PRM repeats phase separation was not observed even when both constituents were present at 500 μm. Droplets ranged in size from ~1 μm to >50 μm and also exhibit liquid characteristics like diffusion of droplet components and coalescence of smaller droplets into larger droplets. Notably, the affinity of a single $SH3_1$/PRM_1 pair used in these studies was quite low with a K_D of 350 μM. While higher affinity interactions may reduce the critical concentration for phase separation they also likely reduce the dynamics of the phase-separated material as measured by the rate of fluorescence recovery [13••]. By contrast to amyloid fiber formation, liquid–liquid phase separation likely relies on (non-covalent) polymerization through relatively weak multivalent interactions, which are constantly being broken and reformed resulting in fluid like properties and exchange of proteins between the protein-enriched and depleted phases. The interplay between multivalency, affinity and concentration

in formation of the phase-separated droplets can be modelled using phase separation principles from polymer chemistry [5••,11,12••,31,32].

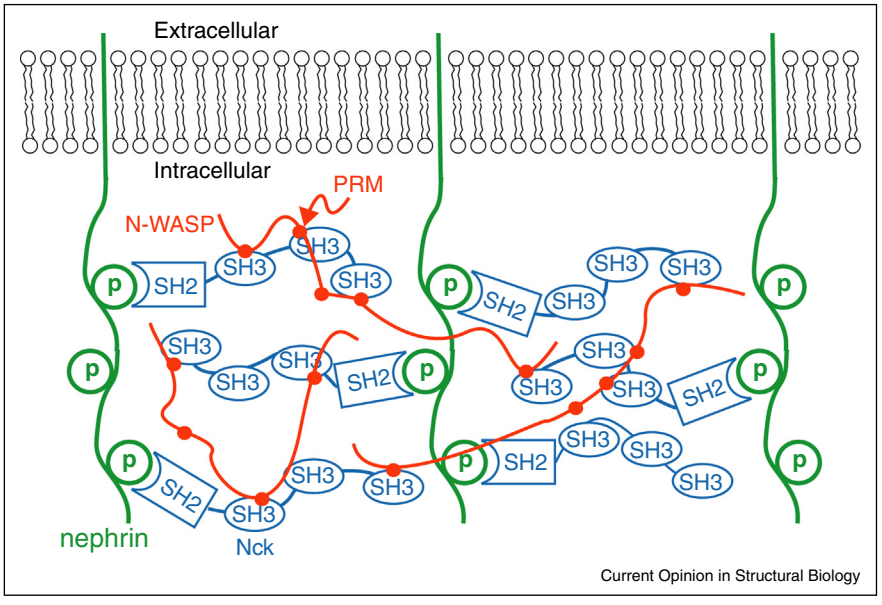
Two-dimensional phase separation or clustering

The multivalent interactions in the preceding example are derived from the cytoplasmic proteins Nck and N-WASP, which promote assembly of actin filaments together with the membrane-bound adhesion receptor nephrin and the Arp2/3 complex [13••,33]. Nephrin has three tyrosines on a long disordered tail; upon phosphorylation these can bind to Nck's single SH2 domain [14]. Nck also has three SH3 domains that bind to PRMs in N-WASP (Figure 2). The multivalent interactions between nephrin, Nck and N-WASP bring nephrin into a phase-separated state. However, since nephrin is membrane-bound, the phase separation occurs in an approximate two-dimensional plane near to the membrane and results in clustering of nephrin in the membrane. Clustering of nephrin then promotes actin filament assembly by the Arp2/3 complex. Formation of clusters is highly cooperative, resulting in a sharp phase transition. Clusters are fluid-like and micron-sized and can coalesce into larger clusters. Notably, two-dimensional clustering has a much lower concentration threshold (can occur at 10^{-7} M) than formation of three-dimensional droplets in solution. Multivalency was again shown to be important, with efficient clustering requiring all three nephrin phosphorylated tyrosines and all three SH3 domains.

Similar clustering is observed for the T cell receptor complex protein LAT (linker for activation of T cells), again involving adapter proteins with multiple SH3 domains and a SH2 domain and multiple phosphotyrosine residues on LAT [21•,34]. Notably, these phase-separated LAT clusters act as a selective solvent for other proteins, enriching for the LAT kinase, ZAP70, and excluding the CD45 tyrosine phosphatase. This likely enhances LAT phosphorylation and amplifies signals from the T cell receptor, thus providing an example of how phase separation may enable signal transduction (Figure 1b).

Membrane clustering appears to be a common mechanism in cellular signaling, which may serve to stabilize active conformations, amplify signals or introduce switch-like behavior. It has been observed for many signaling receptors including EGF [35], Fas/FasL [36] and Eph [37,38]. Other membrane proteins for which clustering has been observed include cadherins [39], GPI anchored proteins [40] and the cystic fibrosis transmembrane conductance regulator (CFTR) [41–45]. Interestingly, clustering of CFTR and the GPI-anchored folate-receptor appear to be cholesterol dependent, implying that clustering in these cases may depend on both membrane phase separation and interactions among multivalent protein interaction regions. In the case of CFTR, membrane clustering may rely on the multivalent protein interactions of critical disordered regions including a long regulatory region (R region) and the C-terminus, which act as hubs to integrate signals from diverse inputs including protein kinase A, protein kinase C, adenosine

Figure 2



Membrane-bound nephrin (green) has three tyrosines, which can be phosphorylated. These phosphotyrosines interact with the SH2 domains of Nck (blue). Nck also has three SH3 domains that bind to the PRMs of N-WASP (red). These multivalent interactions lead to clustering of nephrin within the membrane.

monophosphate-stimulated kinase, several phosphatases, the NHERF-ezrin-actin cytoskeleton complex, the 14-3-3 adapter protein, the SCL26A3/6 transporter, calmodulin and the potassium channels KCa3.1 and ROMK2 [46,47,48•,49]. Recently, a subset of CFTR was shown to cluster with KCa3.1 in response to elevated Ca^{2+} [49]. Notably, both CFTR and KCa3.1 have calmodulin binding regions and the calmodulin binding region of KCa3.1 has been implicated in the KCa3.1 association with CFTR [46,49]. CFTR has been demonstrated to bind to calmodulin in a Ca^{2+} dependent manner, through its disordered R region; furthermore, CFTR channel activity is promoted by high intracellular concentrations of Ca^{2+} [46]. Collectively, these data indicate a role for Ca^{2+} signaling in clustering of CFTR and KCa3.1 channels and activation of the CFTR channel and hint at a link between clustering and CFTR activation.

A role for intrinsically disordered proteins in phase separation

Disordered regions are very common in proteins that phase separate or cluster near membranes [22]. Examples include the disordered regions of CFTR (R region and C-terminus), Ddx4 [5••], Fus [50], the carboxy terminal domain of RNA polymerase II [50,51], TAF15 [52], DYRK3 [53], hnRNPA1 [8], LAF-1 [29], the nephrin tail and the N-WASP PRM containing regions [54]. The majority of these proteins have disordered regions of greater than 100 residues in length. Disordered proteins have characteristics that make them amenable to phase separation. Disorder allows recognition motifs, such as the phosphotyrosines in the nephrin tail, to remain exposed to the modular binding domains as well as the modifying enzymes that add post-translational modifications [55]. Disorder provides the conformational flexibility that is required for the phase-separated droplets to remain fluid. It also enables binding events to remain uncoupled [22], which may be another requirement for keeping droplets fluid. IDRs are very common in signaling proteins, but the role of IDRs in phase separation of proteins involved in signaling from membrane receptors remains largely unexplored. However, the roles of IDRs in proteins such as Ddx4 and Fus, that participate in formation of ribonucleoprotein (RNP) granules, provide insights that likely apply to receptor-mediated signaling puncta. The RNA processing (including transcription, splicing and translation) that occurs in RNP granules is in fact a key component of signaling downstream from receptors, so these proteins can also be viewed as 'signaling' proteins.

What is the mechanistic basis for phase separation of these IDPs/IDRs? Phase separation of Ddx4, involved in the pi RNA pathway and forming the matrix of germ granules [56], depends on a disordered segment of the protein. Within the low complexity, N-terminal disordered region of Ddx4 [57], which can phase separate on its own, are alternating blocks of positively and negatively charged regions [5••]. Scrambling of these charges inhibits phase separation, pointing to electrostatic interactions between charge blocks. In addition to these electrostatic forces, the many phenylalanine residues in Ddx4 have also shown to be crucial. The phenylalanine residues and a large number of RG and GR amino acid pairs suggest that cation–pi [58,59] interactions play a role in phase separation. Thus, multiple electrostatic interactions and multiple cation–pi and/or pi-pi interactions contribute to phase separation of Ddx4. Interestingly, Ddx4 droplets have a lower dielectric constant than bulk water [5••] based on the lower water content in this protein-dense phase, reinforcing the electrostatic cohesion of the droplets.

The disordered N-terminal low complexity (LC) region of the phase-separating protein Fus is highly enriched in G, S, T, P, Q and Y amino acids [50], but devoid of R or K residues or negatively charged residues. There are however many R residues in the C-terminal RGG repeats, suggesting possible formation of cation–pi interactions in phase separation of the full-length Fus [52]. Notably, the LC region is able to phase separate on its own albeit at a higher protein concentration. By contrast to Ddx4, phase separation of the Fus LC region is enhanced at high salt concentrations [50,60], indicating a different driving force behind phase separation. However, the highly repetitive nature of the LC region sequence points to the importance of multivalent interactions in this system as well. These differences in driving forces may be crucial for keeping proteins in distinct phase-separated droplets and may also result in different emergent properties in distinct droplets. As for Ddx4, NMR experiments showed that the chemical shifts of the phase-separated LC region are very similar to shifts observed for the dispersed phase LC region, indicating that there is a high degree of flexibility in the phase-separated state, despite having a much slower rate of diffusion than in the dispersed state.

Just as IDRs play a role in maintaining the fluidity of phase-separated proteins, flexible linkers between modular binding domains may have a similar influence in signaling complexes by allowing the domains to be loosely arranged. Supporting this contention, the precise arrangement of modular binding domains within signaling clusters/phase-separated droplets appears to be highly flexible [12••,13••,54,61,62••]. Studies performed in the yeast mating signaling system, which has not yet been demonstrated to phase separate, indicate that signaling can often be maintained even when domains are swapped from one protein to another within the system, providing robustness in the face of genetic recombination events [61,62••]. This domain arrangement flexibility provides evidence that, within the yeast mating system, geometric constraints are weak and the proteins are not arranged in a highly restricted and ordered fashion; instead they form a more fluid composition suggesting that they also undergo

liquid–liquid phase separation. We speculate that flexible linkers or intrinsically disordered regions may be required for maintaining the fluidity of phase-separated droplets.

Collectively, most of the data are consistent with the proteins within phase-separated states being highly dynamic with rapid exchange of binding interactions. However, some phase-separating proteins including Fus [60], Whi3 [63•], and the Dvl DIX domain [9,64], are able to form fibers of varying stability. These fibrous structures may represent an aging process within the highly concentrated phase-separated droplet. For example, dispersal of Whi3 droplets by addition of salt leaves behind salt-resistant fibers in mature droplets, but not newly formed droplets. However, in some cases these fibers are implicated in function. For example, formation of Dvl2 fibers via its DIX domain has been implicated in Dvl2 puncta formation. The DIX domain likely folds into a compact predominantly β-strand domain structure, which subsequently reversibly polymerizes into long fibers, that are sometimes referred to as signalosomes [9,64]. Mutations that disrupt the ability to form these fibers also block puncta formation and Dvl signaling activity. Similar, filamentous polymers have been observed for other signaling systems involving PB1 (Phox and Bbem1) and SAM (sterile alpha motif) domains, which also form signaling polymers or signalosomes by assembling polymers through head-to-tail interactions [64]. Not all of these filaments are reversible. Some resemble amyloid fibers in terms of structure and in terms of stability [65]. Going forward, it will be interesting to understand how these fibers and spherical puncta are related. Phase separation may precede fiber formation as a highly concentrating step. Although, phase-separated droplets are predominantly considered to be liquid, there is some evidence that substructures and fibrous internal structures can form, particularly in long-lived structures like the nucleolus [66,67].

Conclusions: implications of phase separation for signal transduction

Given the importance of phase separation for RNA processing bodies [22] and evidence of phase separation in signaling puncta below activated receptors [21•], we envision that signals are propagated in various ways in the cell utilizing phase-separated protein states. Signaling controls cellular ion concentrations, expression levels of various proteins and activation of kinases and other enzymes that can post-translationally modify proteins forming the matrix of cellular bodies and puncta, all of which can effect changes in phase-separation behavior [22]. One of the most important outcomes of phase separation for signal transduction is that it can enable switch-like or threshold behavior [11,12••,64] due to the spontaneous and rapid assembly of phase-separated droplets. The weak affinity of individual protein subunits for downstream effectors can be greatly enhanced through avidity in the highly concentrated milieu of the phase-separated body. Even interactions with affinities in the millimolar range can mediate function in a multivalent context [68•]. Phase separation can also create a microenvironment separated from the bulk cytoplasm. Thus, RNP granules concentrate RNA modifying enzymes to make RNA processing more efficient or to protect RNA from modification [22,23]. Receptor-mediated signaling puncta create similar microenvironments, sequestering specific proteins, like activating kinases, while excluding others, such as deactivating phosphatases, to amplify or prolong signals [21•]. Liquid–liquid demixing can also result in emergent properties, which may influence signaling activity [5••]. Phase-separated material may also allow for complex regulation of signal transduction by integrating multiple inputs, similar to integration of multiple inputs by IDR hubs [47]. Phase separation can be regulated by tonicity, pH, phosphorylation, arginine methylation, ubiquitination, and protein expression levels, including by modulating transcription, translation or degradation of granule/cluster components [5••,7,10,24,69,70•]. IDRs likely play a crucial role in phase separation and membrane clustering due to their availability for post-translational modifications and the importance of flexibility for maintaining fluidity in droplets. The frequent occurrence of proteins with multiple modular domains and IDRs implies that phase separation may be very common in signal transduction. Studies to date have already revealed switch-like behavior, the creation of sequestering microenvironments, and signal integration capabilities, but the full implications of phase separation for signal transduction are just beginning to emerge in this nascent field.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was supported by grants to J.D.F.-K. from the Canadian Institutes for Health Research (MOP 114985), Canadian Cancer Society Research Institute (703477) and Cystic Fibrosis Canada (3175). J.D.F.-K. holds a Tier 1 Canada Research Chair in Intrinsically Disordered Proteins.

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