**Autoinhibition in the Signal Transducer CIN85 Modulates B Cell Activation**

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Cite This: https://doi.org/10.1021/jacs.3c09586

**ABSTRACT:** Signal transduction by the ligated B cell antigen receptor (BCR) depends on the reorganization of its intracellular components, such as the effector proteins SLP65 and CIN85 within phase-separated condensates. These liquid-like condensates are based on the interaction between three Src homology 3 (SH3) domains and the corresponding proline-rich recognition motifs (PRM) in CIN85 and SLP65, respectively. However, detailed information on the protein conformation and how it impacts the capability of SLP65/CIN85 condensates to orchestrate BCR signal transduction is still lacking. This study identifies a hitherto unknown intramolecular SH3:PRM interaction between the C-terminal SH3 domain (SH3C) of CIN85 and an adjacent PRM. We used high-resolution nuclear magnetic resonance (NMR) experiments to study the flexible linker region containing the PRM and determined the extent of the interaction in multidomain constructs of the protein. Moreover, we observed that the phosphorylation of a serine residue located in the immediate vicinity of the PRM regulates this intramolecular interaction. This allows for a dynamical modulation of CIN85’s valency toward SLP65. B cell culture experiments further revealed that the PRM/SH3C interaction is crucial for maintaining the physiological level of SLP65/CIN85 condensate formation, activation-induced membrane recruitment of CIN85, and subsequent mobilization of Ca2+. Our findings therefore suggest that the intramolecular interaction with the adjacent disordered linker is effective in modulating CIN85’s valency both *in vitro* and *in vivo*. This therefore constitutes a powerful way for the modulation of SLP65/CIN85 condensate formation and subsequent B cell signaling processes within the cell.

**INTRODUCTION**

Scaffold proteins play an important role in the spatial and temporal organization of cellular processes, and thus their significance for many of the interconnected signaling pathways cannot be overstated. Their efficient use of multiple modular domains and intrinsically disordered regions (IDR) enables the formation of the large macromolecular assemblies that play an important role in nearly all of the signaling pathways known within the cell. Specific functions range from recruiting effectors to specific subcellular locations, providing docking sites for the assembly of higher-order macromolecular structures, and to fine-tune the often weak and transient interactions within these assemblies. In particular, the combination of modular folded domains connected via IDR leads to multidomain proteins with a large potential for internal dynamics, necessary for their many different functions. Consistently, the same scaffold protein can play different roles in separate signaling pathways, depending on differential splicing, post-translational modifications (PTM), and/or the presence of different other effector and scaffold proteins.

The Cbl-interacting protein of 85 kDa (CIN85) is a protein expressed in many different cell types, involved in processes as diverse as cytokinesis, lysosomal degradation of the epidermal growth factor receptor, clathrin-mediated receptor internalization, cell adhesion and cytoskeletal remodeling, and both T cell receptor and B cell receptor (BCR) signaling. In the context of processes associated with BCR signaling, CIN85 was shown to be constitutively associated with Src homology 2 domain-containing leukocyte protein of 65 kDa (SLP65), engaging in promiscuous multivalent interactions between its Src-homology 3 (SH3) domains and SLP65 proline-rich motifs (PRMs). By association with small unilamellar phospholipid vesicles via the N-terminal domain of SLP65, all these transient interactions lead to the formation of...
Recent advances in structural biology have revealed that proteins containing flexible linker regions is autoinhibition. Consequently, a common mode of regulation for multidomain proteins is modulated by interactions of the disordered regions of CIN85, leading to intra- or intermolecular autoinhibition. This is commonly caused by recognition motifs inside flexible linker or tail regions, occupying one of the domains completely until the interaction is perturbed. This results in the domain being able to engage with other effectors and/or become catalytically active. There is evidence by previous studies that CIN85 SH3 domains are able to recognize PRMs within disordered regions of CIN85, leading to intramolecular autoinhibition. Notably, Li et al. have provided indirect evidence of an autoinhibitory interaction mediated by the SH3C domain to the adjacent linker based on isothermal titration calorimetry experiments but did not investigate this further. Because the propensity for LLPS is mainly driven by PTMs, such as phosphorylation at Tyr, Ser, or Thr residues. Consequently, a common mode of regulation for multidomain proteins containing flexible linker regions is autoinhibition. This is commonly caused by recognition motifs inside flexible linker or tail regions, occupying one of the domains completely until the interaction is perturbed. This results in the domain being able to engage with other effectors and/or become catalytically active. There is evidence by previous studies that CIN85 SH3 domains are able to recognize PRMs within disordered regions of CIN85, leading to intramolecular autoinhibition. Notably, Li et al. have provided indirect evidence of an autoinhibitory interaction mediated by the SH3C domain to the adjacent linker based on isothermal titration calorimetry experiments but did not investigate this further. Because the propensity for LLPS is mainly driven by PTMs, such as phosphorylation at Tyr, Ser, or Thr residues. Consequently, a common mode of regulation for multidomain proteins containing flexible linker regions is autoinhibition. This is commonly caused by recognition motifs inside flexible linker or tail regions, occupying one of the domains completely until the interaction is perturbed. This results in the domain being able to engage with other effectors and/or become catalytically active. There is evidence by previous studies that CIN85 SH3 domains are able to recognize PRMs within disordered regions of CIN85, leading to intramolecular autoinhibition.

In this study, we identify a hitherto unknown PRM that predominantly interacts with the CIN85 SH3C domain. Using NMR spectroscopy, we characterize the interaction of the CIN85 SH3 domains with synthetic peptides, also addressing the influence of mutations on the binding. We assign the backbone resonances of the disordered linker containing the motif and investigate SH3:PRM binding via NMR relaxation and translational diffusion experiments in multidomain protein constructs of various lengths. We determined that this SH3:PRM interaction modulates the valency of the CIN85 protein and therefore the extent of interaction with its constitutive binding partner SLP65. Finally, we show the relevance of this interaction in DG75 B cell lymphoma cells for modulating B cell responses to stimulation of the BCR.

**RESULTS**

The Second IDR in CIN85 Contains a Novel PRM That Interacts Preferably with SH3C. We first investigated whether the linker regions in CIN85 were predicted to show deviations from a purely disordered linker. For this, we employed two predictors that are based on flexible regions in high-resolution X-ray structures (DISOPRED3) and backbone flexibility from NMR chemical shifts of IDPs (Dynami). DISOPRED3 scores will be high for highly disordered sequences, while the Dynami order parameter prediction indicates more rigid structures at high values. As displayed in Figure 1A, both predictors were able to distinguish the folded domains (SH3A-C and the coiled-coil (CC) domain) from the disordered linkers. In addition, both predictors show a significant deviation from a purely disordered sequence in the linker region between SH3B and SH3C (residues 162−263). Conserved residues in protein sequences can indicate functional importance, even in intrinsically disordered regions that typically do not show a high degree of conservation. We therefore determined the sequence conservation within the intrinsically disordered linker between SH3B and SH3C by performing a BLAST search starting from the CIN85 Uniprot entry Q96B97-1 (referenced to residues 200−239). The sequence conservation was plotted as bars at the top of each amino acid position. The Second IDR in CIN85 Contains a Novel PRM That Interacts Preferably with SH3C. We first investigated whether the linker regions in CIN85 were predicted to show deviations from a purely disordered linker. For this, we employed two predictors that are based on flexible regions in high-resolution X-ray structures (DISOPRED3) and backbone flexibility from NMR chemical shifts of IDPs (Dynami). DISOPRED3 scores will be high for highly disordered sequences, while the Dynami order parameter prediction indicates more rigid structures at high values. As displayed in Figure 1A, both predictors were able to distinguish the folded domains (SH3A-C and the coiled-coil (CC) domain) from the disordered linkers. In addition, both predictors show a significant deviation from a purely disordered sequence in the linker region between SH3B and SH3C (residues 162−263). Conserved residues in protein sequences can indicate functional importance, even in intrinsically disordered regions that typically do not show a high degree of conservation. We therefore determined the sequence conservation within the intrinsically disordered linker between SH3B and SH3C by performing a BLAST search starting from the CIN85 Uniprot entry Q96B97-1 (referenced to residues 200−239). The sequence conservation was plotted as bars at the top of each amino acid position.
chemical shift perturbations (CSP) in the $^{15}$N-labeled SH3 domains upon titration with a synthetic 14-residue peptide of the sequence $^{219}$FKDKPIKLPRSIE$^{232}$ (see Figure S2). All three domains displayed moderate to weak affinity, with SH3C showing 3- and 5-fold lower dissociation constants ($K_D$) than SH3A and SH3B, respectively ($K_D \sim 0.2$–1.1 mM; see Table 1). This suggested the SH3C domain to be the dominant interaction partner to CIN85-PRM1. We mapped the CSP onto the first model of the NMR structure of SH3C (PDB: 2k9g) in Figure 2A. Binding to the CIN85-PRM1 peptide occurs through the conserved binding site, involving the RT-loop (residues 278–292), the N-Src-loop (residues 300–307), and also the $3_1$-helix (residues 320–322) of SH3C (see Figure S2).

The recognition of PRM by SH3 domains typically depends on the presence of positively charged residues, such as arginines at the motif’s N- or C-terminus, which often form cation−π interactions with conserved tryptophan residues in the SH3 binding interface. In past studies on similar systems, the introduction of R/A mutations was found to be effective in perturbing this type of interaction. We therefore used CIN85-PRM1 mutant peptide binding to the SH3C domain to determine the role of both arginines (R227 and R229) in this interaction. The $K_D$ increased 4-fold for the R227A and 12-fold for the R229A mutant, with complete abolition of binding only after mutating both residues (see Figure 2B, Table 1, and Figure S3). The two arginine residues therefore contribute to the interaction to a different extent, with R229 playing a larger role. Because we knew that this interaction was in part driven by cation−π interactions, we anticipated a net energetic contribution on the order of $-12 \pm 6$ kJ/mol if such an interaction would be disturbed by the mutation. Indeed, we found this difference to be $3.28 \pm 0.20$ kJ/mol for the R227A mutant and $6.32 \pm 1.00$ kJ/mol for the R229A mutant peptides based on their $K_D$’s. The difference in binding energy compared to wild-type peptide was consistent with the loss of a weak cation−π interaction for the R229A mutant, while for R227A, a specific interaction was unlikely, and this residue is more likely involved in nonspecific interactions within the binding interface. We suggest that both R227 and R229 tune the interaction in synergy, with R229 contributing most to the binding affinity, thus being potentially involved in a more persistent, specific interaction. Interpreting these results, one must bear in mind that these differences in binding energy come from NMR titrations of a synthetic peptide with an isolated SH3 domain. In the context of the whole protein, local concentration and/or cooperative effects could increase the actual strength of this interaction considerably. The residue S230 is adjacent to R229 and a known site for activation-induced phosphorylation in CIN85. To investigate the effect of S230s involvement in this interaction, we incorporated a phosphoserine (pS230), a S230D, and a S230A mutation into the synthetic peptide. We observed a similar increase in the $K_D$ for pS230 as for the R229A mutation, while it was not at all affected by the S230A mutation (see Figure 2B and Table 1). This was consistent with a role of this residue in tuning the extent of interaction with CIN85-PRM1 only by post-translational modification while not being involved in the interaction in general. The phosphomimetic mutation S230D led to a much smaller increase in $K_D$, suggesting a specific role of the phosphoryl group in terms of electronegativity and excluded volume. By correlating the CSP of mutated peptides and the wild-type peptide, we also determined that the mode of binding was conserved, and differences in dissociation constants were only due to weakening of the interaction (Figure S5).

### Table 1. Dissociation Constants ($K_D$) of the Binary SH3:Peptide Interactions as Determined by NMR Titrations

<table>
<thead>
<tr>
<th>Domain</th>
<th>Peptide</th>
<th>$K_D$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH3A</td>
<td>$^{219}$FKDKPIKLPRSIE$^{232}$</td>
<td>$0.73 \pm 0.02$</td>
</tr>
<tr>
<td>SH3B</td>
<td>$^{219}$FKDKPIKLPRSIE$^{232}$</td>
<td>$1.09 \pm 0.05$</td>
</tr>
<tr>
<td>SH3C</td>
<td>$^{219}$FKDKPIKLPRSIE$^{232}$</td>
<td>$0.21 \pm 0.01$</td>
</tr>
<tr>
<td>SH3C</td>
<td>$^{219}$FKDKPIKLPRASIE$^{232}$</td>
<td>$0.75 \pm 0.01$</td>
</tr>
<tr>
<td>SH3C</td>
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<td>$2.45 \pm 0.27$</td>
</tr>
<tr>
<td>SH3C</td>
<td>$^{219}$FKDKPIKLAPASIE$^{232}$</td>
<td>$\geq 2.45$</td>
</tr>
<tr>
<td>SH3C</td>
<td>$^{219}$FKDKPIKLPRP8SIE$^{232}$</td>
<td>$2.35 \pm 0.08$</td>
</tr>
<tr>
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<td>$^{219}$FKDKPIKLPRAF8SIE$^{232}$</td>
<td>$0.20 \pm 0.01$</td>
</tr>
<tr>
<td>SH3C</td>
<td>$^{219}$FKDKPIKLPRD8SIE$^{232}$</td>
<td>$0.71 \pm 0.03$</td>
</tr>
</tbody>
</table>

*The error in the fitted $K_D$ values was determined via a bootstrap resampling approach.*

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**Figure 2. Interaction of CIN85 SH3C with CIN85-PRM1 peptides.** (A) Chemical shift mapping of residues inside the SH3C domain exhibiting CSP (colored in shades of green) when titrated with an excess of the wild-type CIN85-PRM1 peptide. Residues for which no assignment was available are colored light blue. The SH3C domain structure used here was the first structure of the NMR ensemble deposited in the PDB as entry2k9g. (B) Bar plots showing the CSP of the residues within the SH3C domain in response to titration with the indicated CIN85-PRM1 peptides. For all the data shown, the molar ligand:protein ratio was chosen to be similar, ranging from 10.8 to 12.9. The secondary structure graph of SH3C on top of this figure was generated from the STRIDE prediction of the PDB entry 2k9g using the SSS-Drawer Python script (https://github.com/zharmad/SSS-Drawer).
**CIN85-PRM1 Forms Helical Structures upon Interaction to SH3C.** To study the potential interaction of the SH3C domain with CIN85-PRM1 in more detail, we needed to assign the linker backbone resonances first. We accomplished the near-complete assignment of the linker region containing CIN85-PRM1 by acquiring three-dimensional 13C-detected spectra normalized to the C-terminal residue I164 for CIN85163−333. The resonances within CIN85-PRM1 were severely broadened in both CIN85163−333 and the R229A mutant. Only upon introducing the R227A/R229A mutation did we observe the SNR increase to the level of the surrounding linker.

Significant CSPs were observed within CIN85-PRM1 (residues 280−285) and the RT-loop (residues 300−306) of the SH3C domain, consistent with the NMR titration results of CIN85-PRM1 peptides to the SH3C domain (see Figures 3D and 2B). Additionally, the secondary structure propensities (SSP) were predicted from the assigned chemical shifts using the ncSPC webserver and showed a distinct propensity for helical structures within CIN85-PRM1 (Figure 3B). Torsion angles predicted using TALOS-N were consistent with a 3_10 helix formed by I224, K225, and L226. This was lost upon introducing the R/A mutations (Figure 3B). We assigned the resonances of the free peptide in order to determine whether the helical structures within CIN85-PRM1 form upon binding or are already present in the free peptide but disrupted by the R/A mutations (Figures S8 and S9). We found no significant propensity for helix in the free peptide and the helical structure thus likely forms through a disorder-to-order transition upon binding to SH3C. This is consistent with known structures of SH3 domains bound to their respective peptides, e.g., the complex between GADS-SH3C and a SLP76-peptide (Figure S10A). For that complex, it was shown that the 3_10 helix within the peptide forms similarly through a disorder-to-order transition.**
Small Bound-State Population of CIN85_{163-333} Based on Local Correlation Times. We further used the TRACT experiment to determine residue-specific apparent rotational correlation times ($\tau_c$) within CIN85_{163-333} and sample the interaction between SH3C and CIN85-PRM1 (Figure 3E). We observed small local correlation times $\tau_c$ for linker residues outside of CIN85-PRM1 and an increase within its core for CIN85_{163-333} ($\tau_c = 4.3$ ns). The R229A mutant showed a distinct decrease in $\tau_c$ of residues within CIN85-PRM1 ($\tau_c = 2.3$ ns), and for the R227A/R229A mutant there was no significant difference to the surrounding linker ($\tau_c = 0.5$–1 ns). Here, we focused on the residues within CIN85-PRM1 with the highest correlation times, as these determine the bound fraction. The smaller correlation times of other residues within the motif can be explained by the individual binding behaviors of the amino acids and the variability in complexes formed through fuzzy interactions. Clearly, even in wild-type CIN85_{163-333} none of the residues of CIN85-PRM1 showed the $\tau_c$ of the SH3C domain, averaging at around 11 ns. Bound fractions were estimated based on maximum local correlation times in CIN85-PRM1 and the average correlation time of the SH3C domain. This was calculated to be 31% for CIN85_{163-333}, 14% for the R229A mutant, and zero for the R227A/R229A mutant. For an isolated SH3 domain at room temperature, global $\tau_c$ values are generally found between 4 and 5 ns.

Arginine Side-Chain Rotational Dynamics Indicate Competition between Both Arginines in CIN85-PRM1. Because the arginine residues were arguably playing a major role in this interaction, we further probed rotational dynamics of the arginine guanidinium groups in CIN85_{163-333} through multiquantum chemical exchange saturation transfer (MQ-CEST).

The SH3C Domain in CIN85-PRM1 is Likely to be Favored over Both Remaining SH3 Domains. One small increase in the apparent $\tau_c$ within the SH3C domain compared to the R227A/R229A mutant where binding is abolished (see Figure 3E).

Effective Concentration Effects Favor the Interaction of SH3C to CIN85-PRM1. For recognition motifs tethered to their receptor, effective concentration ($c_{\text{eff}}$) effects have been shown to have a significant influence on binding.

The SH3C Domain in CIN85_{1-333} Is Autoinhibited by Binding to CIN85-PRM1 In Intramolecularly. In contrast to a disordered tail, a linker tethered on both ends will likely have a decreased entropic chain character. This can have the opposite effect compared to the disordered tail, maximizing the local domain concentration and thereby enforcing an intramolecular interaction. To test whether the population of the bound state was indeed larger in the longer CIN85_{1-333}, we transferred the assignment of the linker region from the truncated CIN85_{163-333} by comparing their $^{15}$N TROSY spectra (Figures S17 and S18) and determined the apparent $\tau_c$ values for CIN85_{1-333} and its R/A mutants (Figure 4B).
residue S230 was used here as a proxy for the apparent \( \tau_c \) of the CIN85-PRM1 core because residues L226-R229 could not be assigned in the \( ^1H-^{15}N \) TROSY spectrum of CIN85-1-333. Indeed, we observed a significantly higher population of the bound state in CIN85-1-333, characterized by a distinct increase in \( \tau_c \) of residue S230 (\( \tau_c^{S230} = 18.3 \text{ ns at 0.5 mM} \)). As for the shorter CIN85-163-333, the R229A and R227A and R229A mutations were effective in abolishing this interaction. In comparison, the median \( \tau_c \) for the three SH3 domains at this concentration ranged between 12.7 and 18.2 ns (Figure S19).

Some residues such as D303 (part of the n-Src loop of SH3C; see also Figure 2B) exhibited values significantly larger than these median values (\( \tau_c^{D303} = 20.9 \text{ ns at 0.5 mM} \)). The elevated \( \tau_c \) observed for S230 can, therefore, be explained by the interaction of CIN85-PRM1 with the binding interface of one of the SH3 domains.

To determine whether the interaction with CIN85-PRM1 was dominated by an intramolecular or intermolecular binding mode, we further analyzed residue-specific \( \tau_c \) values and translational diffusion coefficients \( D_t \) over a wide concentration range for CIN85-1-333 and its R229A mutant (Figure 4C,D). The R229A mutant was chosen as a control because it already sufficiently perturbed the interaction with CIN85-PRM1 (Figure 4B). In CIN85-1-333, we found the ratio of \( \tau_c \) values between residues within CIN85-PRM1 and the SH3 domains to be independent of concentration, proving that the interaction was intramolecular. As expected, the R229A mutant showed small \( \tau_c \) values within CIN85-PRM1 at all concentrations, more similar to those of the surrounding linker. These findings were corroborated by the translational diffusion coefficients (\( D_t \)) determined via N-TRO-STE experiments (Figure 4D). CIN85-1-333 consistently showed higher \( D_t \) values than the R229A mutant did at all concentrations, indicating slower translational motion of the mutant (6%–14% difference, depending on concentration) and consequently a more compact shape of CIN85-1-333 compared to CIN85-1-333-R229A. In addition to the differences between constructs indicating the intramolecular SH3-PRM association, we also observed effects common to both CIN85-1-333 and the R229A mutant. These showed a general concentration dependence of local apparent \( \tau_c \), within the SH3 domains and the global \( D_t \) (Figure 4C,D). We also found the slope of the concentration dependence of \( D_t \) for both constructs to be the same within the experimental uncertainty (Figure S20). This showed the
concentration dependence of these parameters to be independent of the interaction with CIN85-PRM1 and thus common to both constructs. To rule out the possibility that viscosity changes with protein concentration were mainly responsible for the observed differences, we measured relaxation rates of bulk water in the buffer at different protein concentrations and estimated the resulting viscosity changes (Figure S21). The measured dynamic viscosity increased by a factor of 1.32 from pure buffer to a CIN85 concentration of 1.3 mM. However, the median rotational correlation time of the three SH3 domains in CIN85-S230A and the R229A mutant increased by a factor of 1.9–2.3 and 2.0–2.6, respectively (Figure S19). Consistently, we found this change to be a factor of 2.4 and 2.7 for $D_\nu$ (Figure 4D). Therefore, the observed differences in $\tau$ and $D_\nu$ with the concentration cannot be attributed to viscosity changes alone. These differences can be explained by assuming an increasing extent of transient nonspecific protein–protein interactions with concentration, which however do not involve the CIN85-PRM1:SH3 interaction. Thus, in addition to the specific trimerization via the coiled-coil domain that has been described previously, CIN85 SH3 domains can also mediate nonspecific low-affinity oligomerization by themselves.

**CIN85-PRM1 Phosphorylated at S230 Provides an Activation-Induced Release of CIN85 SH3 Domains.** The serine residue at position 230 was shown to significantly weaken the CIN85-PRM1:SH3 association in its phosphorylated state (Figure 2B and Table 1). In addition, it was found to be highly phosphorylated in a multitude of phosphoproteomic studies (Figure S4). In particular, S230 has been shown to be phosphorylated during tonic signaling in B cells and in response to BCR engagement, which is why we decided to assess the signaling function of this residue. We introduced fluorescently labeled full-length citrine (cit)-CIN85 and cit-CIN85-S230A into DG75 B cells expressing no endogenous CIN85. The intact BCR-related signaling machinery made this cell line suitable for studying the effect of the mutation on B cell signaling. The S230A mutation was selected because it did not affect SH3C’s interaction with the corresponding mutant peptide (see Table 1), and it created a mutant that could not be phosphorylated at position S230. It has been shown previously that the multivalent and promiscuous interactions between CIN85 SH3 domains and SLP65 PRMs drive the formation of liquid-like condensates that are a necessary prerequisite for BCR signaling events to occur. Monitoring the propensity for LLPS is therefore a viable readout for the overall BCR-related signaling capabilities of the S230A mutant. Utilizing imaging flow cytometry, we observed a decrease in the percentage of droplet-positive cells and attenuated BCR-induced recruitment to the plasma membrane of the S230A variant compared to wild-type CIN85 (see Figure 5A,B). These findings correlated with compromised BCR-induced Ca$^{2+}$ mobilization in CIN85-S230A-expressing DG75 B cells compared to cells producing the wild-type protein (Figure 5C). This indicated that the modulation of LLPS propensity by phosphorylation at S230 is a probable scenario for efficient BCR-induced signaling via liquid-like condensates, corroborating what has been shown previously by Wong et al.

We further used label-free mass spectrometry to investigate how phosphorylation of CIN85-PRM1 changes the CIN85 interactome. For this purpose, we purified CIN85 from lysates of DG75 B cells expressing either wild-type cit-CIN85 or cit-CIN85-S230A, in both the resting and stimulated state (see Figures S22 and S23). Comparing the abundance of proteins interacting with cit-CIN85 relative to cit-CIN85-S230A, this approach did not reveal any specific protein interacting with CIN85-PRM1 in either the resting or stimulated state. Therefore, we could exclude that a specific interaction was lost upon introducing the S230A mutation. This also indicated that phosphorylated S230 had no specific interaction partner, and its sole function in the context of BCR signaling might be the modulation of the interaction between CIN85-PRM1 and SH3C. Nevertheless, we observed more subtle differences in the interactome of CIN85. We determined decreased abundances of both SLP65 and CIN85 in preparations of the S230A variant. Because we did not detect a difference in

![Figure 5](image-url)
the expression of the mutant compared to the wild type (Figure S24), this can likely be attributed to the reduced propensity for these two proteins to engage in network formation leading to liquid-like condensates (Figure 5A).

**DISCUSSION**

The interaction networks mediated by scaffold proteins such as CIN85 and SLP65 have been shown to be of primary importance for the physiological signaling processes occurring in human and murine B cells. Previous work addressed the assembly of these two scaffolds into liquid-like presignaling clusters that are a prerequisite for the proper function of BCR signaling and enable a rapid cellular response upon BCR engagement. Yet, open questions still exist regarding the necessary modifications in CIN85 and SLP65 to modulate their propensity for undergoing LLPS and to enable a signaling-competent state. The CIN85 protein serves a multitude of different context-sensitive functions in a variety of different cell types, which require finely balanced and tightly controlled regulation. In multidomain proteins, an effective way of providing regulation by the cell is through a SH3-mediated intramolecular association involving IDRs. This recurring theme of autoinhibited SH3 domains has been observed for several different systems in immune cells, including the Nck adaptor protein in T cells and the cytosolic component of the NADPH oxidase p47phox in phagocytes. From the perspective of cellular signaling, regulation by intramolecular interactions has the advantage of being concentration independent and, due to effective concentration effects, needing only moderate nominal affinities to compete with potential intermolecular binding partners.

Based on our findings, we therefore suggest that one of the SH3 domains is autoinhibited by the intramolecular interaction with CIN85-PRM1 in the adjacent linker region. From the nature of the NMR relaxation experiments presented here (Figure 4B,C), we can only definitively say that the bound state is the major populated state, but not whether one or multiple of the domains enforce this interaction synergistically. However, we observed a clear hierarchy of binding affinities of the different SH3 domains to CIN85-PRM1, with SH3C binding the strongest, followed by SH3A and SH3B (Table 1). This hierarchy is also supported by the phylogenetic origin of these domains, as SH3B and SH3C have split from the common progenitor SH3 domain first, while the SH3A domain was later generated via gene duplication of SH3C, making them more similar compared to SH3B. This also reflects the fact that SH3A and SH3B exhibit strikingly dissimilar binding mechanisms to similar peptides. In addition, our analysis of dissociation constants and effective concentrations suggests that SH3C is likely to outcompete SH3A and SH3B (Table 1, Figures S15 and S16). Therefore, based on the data at hand, we argue that CIN85-PRM1 is able to compete with SLP65 PRM’s for binding to SH3C and is fully bound by the domain while the SH3A and SH3B domains are free to engage with SLP65.

The given state of a multidomain scaffold protein like CIN85 is typically determined by the presence or absence of many different nominally low-affinity interactions. The autoinhibitory interaction between the SH3C domain and CIN85-PRM1 is thus a potential candidate for shifting these equilibria toward a signaling-competent state of the CIN85 protein upon engagement of the BCR. Having established the autoinhibition of the SH3C domain *in vitro*, we addressed its potential signaling consequences in the cellular context. Because the promiscuous interactions of CIN85 SH3 domains with SLP65 PRMs drive the LLPS in conjunction with small vesicles, modulating the valency of the CIN85 protein should also have an effect on the propensity for phase separation. Indeed, a study conducted in parallel by our group has investigated the phase separation behavior of CIN85 and SLP65 *in vitro* droplet reconstitution assays and lattice-based computational modeling. One main finding of that study was that the threshold concentration for LLPS was lowered for the CIN851−335-R227A/R229A mutant in which the interaction between SH3C and CIN85-PRM1 was abolished (see Figure S8 in Maier et al.). This can be understood quantitatively only if one assumes the presence of an autoinhibitory interaction, preventing one of the SH3 domains from interacting with SLP65 PRMs. These results are therefore consistent with the proposed autoinhibition of the SH3C domain, as discussed above. The activation-induced phosphorylation of S230, located directly adjacent to CIN85-PRM1 (Figure 1B), has been shown to occur shortly after the stimulation of the BCR. Taking into account the presented evidence of the intramolecular interaction between SH3C and CIN85-PRM1 *in vitro*, we suggest that this motif acts as a "switch" to modulate CIN85’s valency depending on the cellular signaling state. The data from cultured B cells presented here support this hypothesis, as the propensity for LLPS was significantly decreased in the cit-CIN85-S230A mutant cells, where this interaction is expected to be persistently active (Figure 5A). This also suggests a significant population of phosphorylated CIN85-PRM1 in the state of tonic signaling because this difference was already apparent in the nonstimulated cells. Modulating the extent of this interaction could therefore help to maintain the correct level of LLPS in resting B cells and shift the equilibrium depending on their cellular activation state. This in turn can influence the response of these cells to external stimuli, which was evident here from the reduced recruitment of CIN85 to the plasma membrane and the diminished mobilization of Ca²⁺ in response to BCR engagement (see Figure 5B,C).

In conclusion, we propose a mechanism by which the SH3C domain of CIN85 is autoinhibited by an intramolecular interaction to CIN85-PRM1. This interaction is regulated intracellularly through phosphorylation at neighboring residue S230, which upon phosphorylation enables the SH3C domain to engage in interactions with SLP65 and other effectors, promoting the physiological signaling-competent state. We further propose that the modulation of the interaction between CIN85 SH3 domains and CIN85-PRM1 is important for maintaining the preformed signaling clusters of CIN85 and SLP65 to allow for a dynamic and contextual response depending on the cellular activation state.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://doi.org/10.1021/jacs.3c09586.

Experimental section; resonance assignment of flexible linker regions in CIN85 using a combination of ¹H- and ¹³C-detected NMR experiments; determination of intramolecular effective concentrations from HADDOCK-derived complex structures; determination of arginine

**Article**

[https://doi.org/10.1021/jacs.3c09586](https://doi.org/10.1021/jacs.3c09586)

*J. Am. Chem. Soc.* XXXX, XXX, XXX−−XXX
side-chain dynamics using MQ-CEST experiments (PDF)
Mass spectrometric analysis of protein abundances in DG75 B cells (XLSX)

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Funding
This work was supported by the Max Planck Society. We thank the DFG (Deutsche Forschungsgemeinschaft) for support in the framework of the SFB860 project B05. N.R.-G. acknowledges the DFG for research grant RE 3655/2-3. Open access funding provided by Max Planck Society.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Claudia Schwiegk for the expression and purification of the recombinant protein samples used throughout this work and Kerstin Overkamp for the synthesis of the peptides used in the titration experiments. We are grateful to Gogulan Karunanithy and D. Flemming Hansen for providing the MQ-CEST pulse programs and analysis scripts and to Joachim Maier for useful discussions.

■ ABBREVIATIONS

BCR, B cell receptor; cit, citrine; CC, coiled-coil; CIN85, Cbl-interacting protein of 85 kDa; CSP, chemical shift perturbations; IDP, intrinsically disordered protein; IDR, intrinsically disordered region; LLPS, liquid–liquid phase separation; MQ-CEST, multiquantum chemical exchange saturation transfer; MSA, multiple sequence alignment; NMR, nuclear magnetic resonance; PRM, probe-rich motif; PTM, post-translational modification; SH3, Src-homology 3; SLP65, Src homology 2 domain-containing leukocyte protein of 65 kDa; SSP, secondary structure propensities; TRACT, TROSY for rotational correlation times; TROSY, transverse relaxation-optimized spectroscopy.

■ REFERENCES


