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Human 14-3-3 Proteins Site-selectively Bind the Mutational Hotspot Region of SARS-CoV-2 Nucleoprotein Modulating its Phosphoregulation

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Abstract

Phosphorylation of SARS-CoV-2 nucleoprotein recruits human cytosolic 14-3-3 proteins playing a well-recognized role in replication of many viruses. Here we use genetic code expansion to demonstrate that 14-3-3 binding is triggered by phosphorylation of SARS-CoV-2 nucleoprotein at either of two pseudo-repeats centered at Ser197 and Thr205. According to fluorescence anisotropy measurements, the pT205-motif, present in SARS-CoV-2 but not in SARS-CoV, is preferred over the pS197-motif by all seven human 14-3-3 isoforms, which collectively display an unforeseen pT205/pS197 peptide binding selectivity hierarchy. Crystal structures demonstrate that pS197 and pT205 are mutually exclusive 14-3-3-binding sites, whereas SAXS and biochemical data obtained on the full protein-protein complex indicate that 14-3-3 binding occludes the Ser/Arg-rich region of the nucleoprotein, inhibiting its dephosphorylation. This Ser/Arg-rich region is highly prone to mutations, as exemplified by the Omicron and Delta variants, with our data suggesting that the strength of 14-3-3/nucleoprotein interaction can be linked with the replicative fitness of the virus.

Introduction

The 46-kDa nucleoprotein (N) is common for single-stranded RNA viruses, including positive-sense coronaviruses, and is responsible for replication, packaging and storage of viral genomic RNA.¹⁻³ Being one of the most abundant viral proteins, N can reach up to 1% of the total cellular protein in coronavirus-infected cells⁴⁻⁵ and is a major factor of pathogenicity.⁶ Sharing 89.1% sequence identity with SARS-CoV N, SARS-CoV-2 N consists of the structured N-terminal RNA-binding domain⁷, the structured C-terminal dimerization domain,⁸ disordered tails and long interdomain linker (residues 176–246) (Figure 1(a)).⁹⁻¹⁰

The latter contains a key regulatory Ser/Arg-rich (SR) region (residues ~176–210)¹¹⁻¹³ that mediates higher-order oligomerization of N molecules,¹⁴ liquid–liquid phase separation with RNA¹¹⁻¹⁵ and is also a hotspot of viral mutations.¹⁵⁻¹⁷ The SR region of N becomes rapidly phosphorylated by cytoplasmic protein kinases early in infection¹⁴⁻¹⁸⁻²¹, which likely facilitates decompaction of the viral ribonucleoprotein particles,²² whereas at the later infection stages phosphorylation is substantially reduced²¹⁻²³. Since kinase inhibitors considerably
decrease viral replication and titers, phosphorylation of N is critical for the virus fitness. Specifically, phosphorylation triggers N binding to 14-3-3 – the multi-functional phosphopeptide-binding protein–protein interaction hub represented by seven isoforms in human (β, γ, ε, ζ, η, σ, τ). Upon binding to their specifically phosphorylated protein partners, dimeric 14-3-3 inhibits their dephosphorylation, thereby regulating functional activity, protein–protein interactions and intracellular distribution.

14-3-3 proteins have a widely recognized role in replication of many viruses. 14-3-3 isoforms are amongst the top 1% of highest-expressed proteins in a number of tissues including the brain, lungs and gastrointestinal system that are particularly

**Figure 1.** SARS-CoV-2 N recruits human 14-3-3 using either 197 or 205 phosphosites. a. Schematic representation of N; NTD and CTD, interdomain linker, Ser/Arg-rich region are labeled, Ser197 and Thr205 phosphorylation sites are marked by red circles. b. Sequence alignment of the SR-regions of SARS-CoV-2 and SARS-CoV N proteins, with the phosphosites of interest highlighted in red and potential 14-3-3-binding sites in bold. MERS-CoV N sequence is shown for comparison. Note that SARS-CoV N contains only the first potential 14-3-3-binding site, while MERS-CoV N contains only the second site. c. Phosphorylation-related modification of SARS-CoV-2 N variants analyzed by Phos-tag and SDS-PAGE. d. SEC profiles showing different efficiency of 14-3-3 binding with SARS-CoV-2 N containing either nhpS197 or nhpS205. e,f,g,h. Human 14-3-3 (80 μM) co-elutes with SARS-CoV-2 N (50 μM) containing either nhpS197 (e) or nhpS205 (f) but not with unphosphorylated SARS-CoV-2 N (g) (50 μM) during SEC, as analyzed by SDS-PAGE of the fractions collected. Elution of 14-3-3 alone (h) is shown for reference. Mass markers (left) and protein positions (right) are indicated. Position of the 14-3-3:N complex on the elution profile is indicated by dashed rectangles. SEC runs were carried out at 220 mM NaCl in the samples and running buffer.
susceptible to coronavirus infection. A detectable up-regulation of some 14-3-3 isoforms upon SARS-CoV-2 infection was reported. According to immunofluorescence, immunoprecipitation, siRNA silencing and kinase inhibition data, SARS-CoV N undergoes phosphorylation-dependent association with human 14-3-3 in the cytoplasm of the transfected COS-1 cells, which controls nucleocytoplasmic shuttling of N. Upon silencing of 14-3-3 by siRNA, N accumulated in the nucleus.

In previous work, we have shown that phosphorylation of the SR region enables SARS-CoV-2 N recognition by human 14-3-3 proteins, leading to a 2:2 complex. The use of truncated N variants narrowed the binding site to SRNpS197-TP, conserved in SARS-CoV and SARS-CoV-2 (Figure 1(b)). However, whether other 14-3-3-binding sites exist and the structural basis for 14-3-3/N interactions remained elusive.

Production of specific phosphorylated forms of N is challenged by the dense distribution of phosphorylatable residues (Figure 1(b)), resulting in heterogeneously phosphorylated N forms when incubated with kinases. To ensure specific phosphorylation at selected residues, here we utilized the genetic code expansion approach. We identified two phosphorylated pseudo-repeats centered at Ser197 or Thr205 in SARS-CoV-2 N (Figure 1(b)) that individually are sufficient to trigger 14-3-3 binding of the full-length SARS-CoV-2 N. Using high-throughput fluorescence anisotropy, we determined the affinities of both phosphomotifs to all seven human isoforms of 14-3-3, which is identical to the SARS-CoV-2 N counterpart, i.e., Ser197/C2418 and is the best candidate 14-3-3-binding motif besides Ser197.

To test whether phosphorylation at Thr205 was sufficient for N to bind 14-3-3, we expressed full-length SARS-CoV-2 N with nhpSer at this position. Phos-tag gels confirmed near homogenouse modification (Figure 1(c)) and indeed this phosphosite alone was able to trigger phosphorylated 14-3-3 binding as supported by its co-elution with 14-3-3 on SEC (Figure 1(d, f–h)). In fact, it bound human S197L mutant showed a pronounced phosphorylation-dependent interaction with 14-3-3 (Supplementary Figure 2(b–f)), indicating the presence of alternative 14-3-3-binding sites. The SR region of SARS-CoV-2 N contains a pseudo-repeat around Thr205 (SRGTSP), which has a sequence similar to the Ser197 site (SRNSTP), it undergoes phosphorylation in vivo4,16 and by PKA in vitro, and is the best candidate 14-3-3-binding motif besides Ser197.

Results
Identification of alternative 14-3-3-binding sites within SARS-CoV-2N

The key 14-3-3-binding site of SARS-CoV-2 N was proposed to center at pSer197, which is conserved also in the SARS-CoV N protein (Figure 1(b)). To test this hypothesis directly, we first sought to obtain N protein phosphorylated exclusively at the Ser197 position. Since enzymatic treatment always yielded multiply phosphorylated protein (Figure 1(c)), we utilized genetic code expansion to site-specifically install phosphoserine at a genetically programmable TAG stop codon. Although phosphoserine was successfully incorporated to generate SARS-CoV-2 N pS197 protein, confirmed by the discrete upward shift on a Phos-tag gel electrophoresis, we noticed considerable spontaneous dephosphorylation (Figure 1(c)). Therefore, for further analysis we produced the SARS-CoV-2 N protein with a non-hydrolyzable analog of phosphoserine, phosphonomethylalanine (nhpS197), inserted in place of the TAC197 codon, which caused the desired shift on Phos-tag gel with a negligible amount of unphosphorylated protein (Figure 1(c)). This approach was inspired by effective imitation of phosphoserine by nhpSer in previous 14-3-3 binding studies.32,34

On SEC, human 14-3-3 co-eluted with SARS-CoV-2 N nhpS197, but not with unphosphorylated SARS-CoV-2 N, confirming the formation of the phosphorylation-dependent complex (Figure 1(d, e)) and the role of S197 phosphorylation in recruiting 14-3-3. In accordance, the SARS-CoV N protein, which shares the pS197 site with SARS-CoV-2 N (Figure 1(b)), similarly associated with 14-3-3 in a phosphorylation-dependent manner (Supplementary Figure 1). We then asked if the natural S197L mutation of a SARS-CoV-2 N variant identified in 202035 which cannot be phosphorylated at the position 197, would preclude N from interacting with 14-3-3? The S197L mutant was co-expressed with PKA for phosphorylation and then purified similar to the wild-type protein. As a control we produced unphosphorylated S197L protein, and differences in their phosphorylation status were confirmed by Phos-tag (Supplementary Figure 2(a)). Surprisingly, the S197L mutant showed a pronounced phosphorylation-dependent interaction with 14-3-3 (Supplementary Figure 2(b–f)), indicating the presence of alternative 14-3-3-binding sites. The SR region of SARS-CoV-2 N contains a pseudo-repeat around Thr205 (SRGTSP), which has a sequence similar to the Ser197 site (SRNSTP), it undergoes phosphorylation in vivo4,16 and by PKA in vitro, and is the best candidate 14-3-3-binding motif besides Ser197.
CoV-2 N are alternative 14-3-3-binding sites within the regulatory SR region.

Affinity profiles of Ser197 and Thr205 phosphopeptides towards human 14-3-3 isoforms

Synthetic phosphopeptides WSSRNpS<sup>197</sup>TPGSS and WSSRGpT<sup>205</sup>SPARM, each bearing an added tryptophan residue for more accurate quantification, were labeled by fluorescein 5-isothiocyanate (FITC) and used to determine binding affinities against all seven human 14-3-3 isoforms by fluorescence anisotropy. For this, homotopic MBP-tagged full-length 14-3-3 constructs were used to obtain highly pure protein in an efficient manner. Representative SEC-MALS analysis of MBP-14-3-3 confirmed that the MBP fusion did not disrupt the functional dimeric state of 14-3-3 (Supplementary Figure 3).

Peptide binding to 14-3-3 was saturable and specific, and the MBP tag did not change the $K_D$ (Figure 2(a)). The same FITC-labeled phosphopeptide failed to bind to noncognate protein lysozyme; likewise, free FITC did not show significant binding to MBP-14-3-3 (Figure 2(a)). In addition, the FITC-peptide complexes with 14-3-3 could be disrupted by titration with either the corresponding unlabeled peptides or the canonical phosphopeptide from unrelated human protein HSPB6 (pB6) (Supplementary Figure 4). Therefore, the chosen N peptides bind specifically to the common peptide-binding grooves of 14-3-3.

Both SARS-CoV-2 N phosphopeptides showed saturable interaction with all seven human 14-3-3 isoforms, enabling $K_D$ determination (Figure 2(b) and c), Supplementary Figure 5). The average affinities of 14-3-3 to the SARS-CoV-2 N phosphopeptides were somewhat lower than to the pB6 peptide, yet were in a comparable range.

Figure 2. Affinity and selectivity of SARS-CoV-2 N phosphopeptide binding to the human 14-3-3 proteins. a. Fluorescence anisotropy titration data for 14-3-3γ or MBP-14-3-3γ binding to the FITC-labeled SARS-CoV-2 N pT205 peptide. The corresponding apparent $K_D$ values (mean ± SD, n = 3) are shown. Titration of the same peptide by lysozyme and titration of free FITC by MBP-14-3-3γ are shown as controls. b, c. Titration of FITC-labeled SARS-CoV-2 N phosphopeptides pT205 (b) or pS197 (c) by the seven human 14-3-3 isoforms. The data (mean ± SD, n = 3) are color-coded with respect to the 14-3-3 isoforms; fitting curves and the $K_D$ values at equilibrium are found in Supplementary Figure 5. In a-c, background anisotropy was subtracted from all values. d. Affinity profiles of the SARS-CoV-2 N phosphopeptides versus 14-3-3 isoforms shown as heatmap gradients according to the $K_D$ scale on the right. The source $K_D$ values are found in Supplementary Table 1. e, f. Affinity profiles presented as $-\log K_D$ values (e) or selectivity coefficients (f) are correlated with the proteome-wide affinity hierarchy of the 14-3-3 isoforms represented as average ΔΔG values for the individual isoforms (indicated by asterisks). Coefficient of determination $R^2$ and Pearson’s $r$ coefficient characterizing the linear correlations are indicated.
micromolar range (Supplementary Table 1 and Figure 5). For each SARS-CoV-2 N peptide, we detected a remarkable peptide-affinity trend following the general peptide-affinity hierarchy of 14-3-3 isoforms reported recently (Figure 2(d and e)). For the pT205 peptide, the binding affinity to 14-3-3 decreased from gamma (γ) > eta (η) > beta (β) > epsilon (ε) > zeta (ζ) > tau (τ) > sigma (σ); the order for the pS197 peptide was: β > γ > η > ζ > τ > ε > σ (Figure 2(d)). As a result, transformation of the Kₐ values into -logKₐ showed that both trends correlate well with the interactome-wide affinity hierarchy of the 14-3-3 isoforms (Pearson’s r = 0.87 for pS197 and r = 0.93 for pT205, Figure 2(e)).

The affinity profiles of 14-3-3 for the two SARS-CoV-2 N peptides were significantly shifted and nonparallel to each other. Due to the shift, the affinity of the weakest pT205 peptide binder, 14-3-3σ (Kₐ ∼ 33 μM), was still ~2 times tighter than for the strongest pS197 binders, 14-3-3β/γ/η (Kₐ ∼ 70 μM). Overall, the 14-3-3 family covered affinities to SARS-CoV-2 phosphopeptides differing by an order of magnitude (~10-fold Kₐ ratio between the weakest (14-3-3-σ/pS197) and the strongest (14-3-3-β/PT205) pair), despite the very similar sequences of the peptides and the absolutely conserved 14-3-3 grooves. The average affinity of human 14-3-3 proteins to the studied SARS-CoV-2 phosphopeptides (Kₐ ∼ 50 μM) is roughly in the same order of magnitude as the average affinity of twenty-three unrelated peptides analyzed previously (Kₐ ∼ 45 μM). This suggests viable competition of the SARS-CoV-2 N phosphopeptides with the host cell phosphopeptides for the 14-3-3 binding, especially with the weaker interacting portions of the interactome.

**Selectivity trend of 14-3-3 isoforms to SARS-CoV-2 N phosphopeptides**

Curiously, the affinities to all 14-3-3 isoforms were consistently tighter for the pT205 than for the pS197 peptide (4.3-fold ratio of the average Kₐ values) (Supplementary Table 1). Moreover, we found a remarkable selectivity trend reflecting a linear correlation of the Kₐ ratio for the 14-3-3 isoforms with their general affinity hierarchy (R² = 0.84, Pearson’s r = 0.93, Figure 2(f)). In other words, not only does 14-3-3σ turn out to be the strongest binder, it is also the most selective isoform with respect to the SARS-CoV-2 phosphopeptides pS197 and pT205 (the Kₐ/197/Kₐ/205 ratio of ~7), while 14-3-3σ is the weakest and simultaneously the least selective (Figure 2(f)). Yet, even for 14-3-3σ the affinity to the pT205 peptide was ~3 times tighter than for the pS197 peptide. This observation was totally unexpected given the very similar sequences of both peptides, implying that the corresponding protein-peptide complexes feature different interfaces and chemical contacts. To understand the molecular basis of these phenomena, we crystallized 14-3-3σ complexes with both phosphopeptides and determined structures by X-ray crystallography at high resolution (Supplementary Table 2).

**Crystallography explains the selectivity of 14-3-3 for SARS-CoV-2 N phosphopeptides**

X-ray structures of 14-3-3σ with both peptides had one 14-3-3 dimer in the asymmetric unit, where each protomer was occupied by the target phosphopeptide. Eight out of ten pS197 phosphopeptide residues and all ten residues for the pT205 phosphopeptide are clearly defined in electron density, revealing key protein/peptide interactions. Residues at +1 and −1 positions with respect to each phosphosite are H-bonded to the side chains of the conserved 14-3-3σ residues Asn175 and Asn226, while hydroxyl groups of both Thr198 and Ser206 of the peptides (position +1) are identically involved in H-bond formation with Asn175 and the conserved Lys122 residue of 14-3-3σ (Figure 3(a, b) and Supplementary Figure 6). An additional H-bond in the case of the pS197 peptide between the side chain of its Asn196 residue and Asn226 of 14-3-3σ is replaced by the additional hydrophobic contact in the case of the pT205 peptide, formed between the methyl group of phospho-Thr205 residue and the side chain of 14-3-3σ’s Val178. In contrast to the nearly equivalent central six-residue segments of both peptides (Cα RMSD = 0.7 Å), crystal structures revealed significant differences in their C-terminal regions. In the case of the pS197 peptide, we could not detect any stabilizing in-trans interactions after Pro199. The lack of defined electron density for the last two Ser residues of this peptide indicate flexibility and hence inability to form stabilizing interactions (Figure 3(a)). On the contrary, the pT205 peptide was additionally stabilized in-trans by i) the hydrophobic contacts of its Ala208 with Val46 of 14-3-3 and Met210 with Phe119 and Ile168 of 14-3-3, ii) the H-bonding interaction between the peptide’s carbonyl of Ala208 with the side chain of Ser45 of 14-3-3σ and, most importantly, iii) the salt bridge between the peptide’s Arg209 with Glu14 of 14-3-3σ (Figure 3(b)). Interestingly, Glu14 is absolutely conserved in all human isoforms of 14-3-3 (Supplementary Figure 7). Therefore, the salt bridge interaction involving Arg209 is likely shared by all 14-3-3 isoforms and, together with factors i)-ii), confer the consistently higher affinity of the pT205 peptide versus pS197 peptide to 14-3-3 proteins, as observed biochemically (Figures 1 and 2).

These peptides of the same SARS-CoV-2 N polypeptide chain cannot simultaneously occupy both grooves of the 14-3-3 dimer, as the pS197 and pT205 phosphosites are only 7 residues apart (the inter-phosphate distance is 20 Å). For
bidentate binding to 14-3-3, at least 15 extended residues are needed to sufficiently separate the phosphosites (e.g., 22 in the 5N6N structure) and cover the required ~35 Å distance between the two binding sites of the 14-3-3 dimer (Figure 3). While a mixed pS197/pT205 binding within the 2:2 SARS-CoV-2 N/14-3-3 complex cannot be excluded, binding of two pT205 motifs to the 14-3-3...
3 dimer would be thermodynamically preferred, unless mutations in the SR region change this selectivity. Such bideterminate 14-3-3 dimer binding using either of these two phosphosites is expected to increase the affinity in the context of the full-length proteins ~10-fold.26

**Structure-based prediction of effects of the N mutations on 14-3-3 binding**

The conformation of the 14-3-3-bound peptides allowed us to rationalize the effect of N mutations present in different coronavirus variants (Table 1) by predicting their influence on 14-3-3 binding. First, due to the phosphorylation-dependent nature of 14-3-3 binding, the S197L and T205I mutations of N simply disqualify the corresponding site. Interestingly, the S197L mutation has a statistically mild outcome.35 The T205I mutation, present in multiple variants of SARS-CoV-2, has either mild outcome or co-occurs with multiple mutations in other SARS-CoV-2 genes (https://viralzone.expasy.org/9556), making predictions about its physiological effect challenging. The P199L mutation can decrease the affinity to 14-3-3 at the pS197 site because this position is canonically occupied by Pro,39 but does not compromise 14-3-3 binding at the pT205 site. The S194L mutation, associated with severe COVID-19,35 can site-specifically strengthen 14-3-3 binding due to the additional hydrophobic contacts between Leu194 and a leucine residue of 14-3-3 facing this position in the crystal structure (Figure 3(d)). The widespread combined R203K/G204R mutation present in at least several variants-of-concern (e.g., Alpha, Gamma, Omicron) is expected to enhance phosphorylation of N24 and affect its complex with 14-3-3. While the R203K mutation is expected to slightly decrease the in-cis contact with the phosphate group, the Arg204 side chain can reach the conserved Asp225 of 14-3-3 to make a salt bridge (Figure 3(e)). The S202R mutation has the most profound effect on the efficiency of N to package RNA and produce new viral particles,16 with at least two predictable effects on the 14-3-3 level. First, the S202R mutation can enhance phosphorylation of Thr205, at least by basophilic protein kinases (by forming a stronger canonical sequence RRXT instead of just RXT40). Second, it converts the SRGpTSP 14-3-3-binding site from suboptimal to canonical (RSGpTSP),39 which would strongly promote 14-3-3 binding (Figure 3(f)). The effect of the R203M mutation associated with the Delta variant is difficult to predict at the level of 14-3-3 binding, because this mutation would first interfere with Thr205 phosphorylation in the SMGT205SP context, at least by basophilic protein kinases.

| Mutation | Disease severity | Ref. | Predicted effect on interaction with 14-3-3 (this work) | SARS-CoV-2 name** |
|----------|------------------|------|------------------------------------------------------|------------------|
| S194L    | Severe disease   | 35   | Can be enhanced due to hydrophobic interaction with Leu229* | B.1.1.298***     |
| S197L    | Mild disease     | 35   | None at the S197 site                                 | A.2.4            |
| P199L    | Uncertain/mild   | 58   | Weakening with the S197 site, by compromising the 14-3-3 motif | B.1.526 (Iota)   |
| S202R    | More transmissible, 166-fold higher infectious virus titers | 16  | Significantly improved owing to rectified 14-3-3-binding motif | B.1.526 (Iota)   |
| R203K/G204R | Severe disease, augmented viral replication, increased N phosphorylation | 24  | Enhanced due to predicted Arg204:Asp225* salt bridge | B.1.1.283 (Gamma), B.1.1.28 (Alpha), B.1.1.298*** |
| R203M    | More transmissible, 51-fold higher infectious virus titers | 16  | Uncertain: inhibited via hampered N phosphorylation, neutral due to loss of Arg203 canonical interactions but new potential hydrophobic interaction of Met203 and Leu229* | B.1.617.2 (Delta), B.1.617.1 (Kappa) |
| T205I    | Uncertain/mild   | 59   | None at the T205 site                                 | B.1.351 (Beta), B.1.427 (Epsilon), B.1.429 (Epsil) B.1.525 (Eta), B.1.214.2 |

* Human 14-3-3α residue numbering.
** For variants-of-concern and variants-of-interest, as defined by the World Health Organization, Greek letters are given in parentheses.
*** In B.1.1.298, co-occurrence of S194L and R203K/G204R has been found with high frequency.
14-3-3 occludes the SR region of N and inhibits its dephosphorylation

Unstructured regions of SARS-CoV-2 N restrict its ability to crystallize, therefore we used small-angle X-ray scattering (SAXS) to gain insight into the spatial organization of N in complex with 14-3-3. To make sure that only particles corresponding to the complex were analyzed, we used SEC-SAXS to separate a mixture of phosphorylated N with an excess of 14-3-3γ and ensure scattering data were collected on the intact complex only. This setup also enabled to control the aggregation state of the system, especially important given the inherent higher-order self-association propensity of N. Independent MALS analysis assisted in validating the 2:2 complex stoichiometry (150.8 kDa; expected \( M_w \) is 148.3 kDa) and in selecting the representative region for averaging SAXS curves for the complex (Figure 4(a)).

Complex particles had average \( R_g \) of \( \sim 5.5 \) nm and maximal dimensions of \( \sim 20 \) nm (Figure 4b, Supplementary Table 3). Kratky plots confirmed the presence of folded bodies organized in a non-spherical shape, connected by flexible linkers (Figure 4(c)). To model this complex, we used available atomistic models of dimeric 14-3-3γ as well as of the NTD, dimeric CTD and the short \( \alpha_1 \)-helix in the interdomain linker of SARS-CoV-2 N. Flexibility of IDRs has been confirmed in different N constructs by NMR. We used the N S197L construct because it featured a minimal number of vector-derived residues and contained the major 14-3-3-anchoring pT205 motif in both N chains. While keeping the crystallographic 14-3-3/pT205 core, the complex models revealed that 14-3-3γ occludes the SR region of N and inhibits its dephosphorylation. The presence of human 14-3-3γ has indeed slowed down the phosphatase reaction by > 2-fold, producing a considerable inhibition of SARS-CoV N dephosphorylation in the course of 4 h phosphatase treatment (Figure 4(f, g)). Complete inhibition is likely explained by the presence of N phosphosites beyond the 14-3-3-protected region and by the dynamic nature of the 14-3-3/pN complex indicating that 14-3-3 binding modulates the phosphoregulation of N.

Discussion

Unlike mutations in S protein, that can often be rationalized via their direct effect on viral entry, the effects of mutations in N remain largely unknown. Curiously, the majority of the widespread N mutations beneficial for the virus are mapped to a very small segment, residues 194–205 (Figure 1a, Table 1). In fact, all variants-of-concern defined by the World Health Organization feature at least one mutation in the 199–205 subregion, which coincides with the maximum Shannon entropy in the SARS-CoV-2 genome centered at these positions. Unfortunately, this mutational hotspot SR region of N is disordered, which reduces our ability to deconvolute the underlying effects of relevant mutations at the structural and functional level.

Recent work using the viral-like particles to assess evolved SARS-CoV-2 N variants has shown that the S202R and R203M mutations vastly improve RNA packaging mechanisms and production of higher titers of infectious virions (166- and 51-fold, respectively). Since the improved replicative fitness of the virus correlates well with the increased levels of N phosphorylation, factors promoting N phosphorylation or postponing its dephosphorylation appear particularly relevant for the success of the virus. Noteworthy, 14-3-3 proteins play a well-recognized role in both the replication of many viruses and in protecting their bound phospho-clients from dephosphorylation, which is likely the case for phosphorylated SARS-CoV-2 N when bound to 14-3-3.

We have shown that multiply phosphorylated SARS-CoV-2 N bearing several physiologically-relevant phosphosites can be successfully reconstituted in vitro to induce its interaction with 14-3-3. Our present study identified that 14-3-3 site-selectively recognize two adjacent phosphorylated pseudo-repeats centered at Ser197 and Thr205 of SARS-CoV-2 N. Importantly, this was
Figure 4. In complex with phosphorylated N, 14-3-3γ occludes part of the SR region and inhibits N dephosphorylation. 

a. SEC-MALS profiles of 14-3-3γ or its mixture with the phospho-S197L mutant. Average $M_w$ values and polydispersity indices ($M_w/M_n$) for the peaks are indicated. Highlighted is the region used for structural analysis of the complex based on online SAXS.

b. Pairwise distance distribution function for the complex.

c. Dimensionless Kratky plot for the complex, compared with that for a rigid sphere. The bell-shaped peak and a shoulder corresponding to folded parts of the complex are indicated by arrows.

d. One of the possible conformations of the 2:2 assembly based on CORAL modeling. SARS-CoV-2 N polypeptides are shown by pink and wheat colors, 14-3-3γ dimer is shown by tints of cyan, portions of the interdomain linker of N masked by 14-3-3γ grooves are shown by red, including the 14-3-3γ-bound pT205 phospho-motifs (red spheres in the model) matching those in our 7QIP structure. The foldons of N are named. The regions of N masked by either pS197- or pT205-bound 14-3-3γ are highlighted by red font and ovals on the scheme below. Small yellow circles indicate phosphorylatable residues.

e. The fit of the CORAL model presented on panel d to the SAXS data, with the associated residuals ($\Delta\sigma$).

f. Kinetics of dephosphorylation of phosphorylated SARS-CoV N by shrimp alkaline phosphatase in the absence or presence of human 14-3-3γ, analyzed by Phos-tag SDS-PAGE. Positions of proteins are indicated by arrows.

g. Densitometric analysis of the gel shown on panel f reveals the effect of 14-3-3γ binding on repartition of SARS-CoV N between the phosphorylated (pN) and dephosphorylated (N) forms.
done in the context of full-length proteins and could only be achieved using the latest genetic code expansion approaches enabling site-specific incorporation of phosphoserine and a non-hydrolyzable analog thereof.\textsuperscript{32–33} Switching to the protein-peptide binding format, we used fluorescence anisotropy to show that both SARS-CoV-2 N phosphopeptides display the characteristic peptide-affinity profiles to the human 14-3-3 isoforms.\textsuperscript{28} Unexpectedly, the pT205 fragment interacted with all seven 14-3-3 protein isoforms with a consistently tighter affinity than the pS197 fragment. Moreover, the interaction of the pT205 peptide with the weakest isoform, 14-3-3\(_{3\gamma}\), was still stronger than the interaction of the pS197 peptide with the strongest isoform, 14-3-3\(_{3\sigma}\) (Figure 2).

Most importantly, we identified the remarkable selectivity profile for the 14-3-3 family, a phenomenon that, to the best of our knowledge, has not been previously recognized. This selectivity trend correlated well (\(R^2 = 0.84; r = 0.93\)) with the general peptide-affinity hierarchy of the 14-3-3 isoforms.\textsuperscript{28} Accordingly, not only the strongest peptide binder, 14-3-3\(_{3\gamma}\), binds the pT205 peptide stronger, but its selectivity to this peptide over the pS197 peptide is more pronounced than in the case of generally the weakest isoform, 14-3-3\(_{3\sigma}\) (Figure 2(f)).

To explain the established binding preferences of 14-3-3 proteins toward one of the apparently similar phosphopeptides, we determined crystal structures of 14-3-3\(_{3\sigma}\) with both peptides (Figure 3). The central six-residue portions of the peptides indeed form roughly equivalent contacts with 14-3-3. By contrast, specific stabilizing interactions beyond the central region were observed only in the case of the pT205 peptide. The pS197 and pT205 fragments are mutually exclusive, because their simultaneous placement within the 14-3-3 dimer is sterically constrained (Figure 3(c)). Our model of the 14-3-3 interaction with N based on SAXS data strongly indicated that the complex formation occludes the regulatory SR region of N, i.e., \(\sim\)20–25 residues surrounding each 14-3-3-binding phosphosite, either 197 or 205 (Figure 4(a–e)). Given that > 10 potential phosphosites are affected, this would result in measurable inhibition of N dephosphorylation by 14-3-3. We’ve tested this hypothesis showing that dephosphorylation of phosphorylated SARS-CoV N is indeed inhibited in the presence of 14-3-3\(_{3\gamma}\) (Figure 4(f, g)). This is in line with previous observations where 14-3-3 protected other phospho-client proteins from dephosphorylation\textsuperscript{44–45} and suggests that 14-3-3 binding modulates the phosphoregulation of N.

SARS-CoV-2 N fragments 193–200 and 201–210 co-crystallized with 14-3-3 provide a valuable structural framework for analyzing the effect of the widespread mutations and testing new hypotheses. Collectively, our analyses based on structural, interactomic and bioinformatic data support the hypothesis\textsuperscript{17} that N mutations improving SARS-CoV-2 fitness positively affect its binding to 14-3-3 (Figure 3). Affecting N dephosphorylation, 14-3-3 binding can promote N functions relying on phosphorylation.\textsuperscript{20–23} The SR region of N masked by 14-3-3 is flanked by regions 179–184 and 217–222 that appear to drive amyloid aggregation of N in the course of liquid–liquid phase separation in the presence of RNA\textsuperscript{46}. Because phase separation is important for viral replication and is affected by N phosphorylation\textsuperscript{15,21}, phosphorylation-mediated 14-3-3 binding can act as another layer of regulation that is targeted by the widespread mutations. While this warrants further investigation, we note that 14-3-3 binding, mediated by structurally disordered segments containing phosphorylation sites, emerges as a common feature in virus-host interactions.\textsuperscript{28–29,30,32,47} Given the high biomedical relevance of the 14-3-3/N interaction, its targeting by small molecules, i.e., an increasingly promising approach in the case of many 14-3-3/client complexes,\textsuperscript{48} can be helpful in creating novel therapeutics against coronavirus and other related diseases.

Materials and Methods

Peptides and chemicals

SARS-CoV-2 N peptides phosphorylated at Ser197 (WSSRNpSTPGSS) or Thr205 (WSSRGpTSPARM) were synthesized by Severn Biotech (UK). The phosphopeptide of human HSPB6 (pB6: WLRRApSAPLPGLK) was obtained as previously described.\textsuperscript{36} Expected and experimentally determined masses of the peptides are listed in Supplementary Table 4. FITC was from Sigma-Aldrich (cat no. F7250). Phos-tag acrylamide was purchased from Wako Chemicals (AAL-107). Lysozyme from hen egg white was from Roche (cat no. 10837059001). Shrimp alkaline phosphatase (#EF0511, Fermentas) was kindly provided by Dr. Michael Agaphonov. All common chemicals were of the highest purity and quality available, and all solutions were made with milliQ grade water.

FITC labeling of the SARS-CoV-2 N phosphopeptides

SARS-CoV-2 N peptide labeling was achieved in 0.1 M carbonate buffer, pH 9.0, by mixing a 5 mg/ml solution of the peptide with 1/10 volume of the FITC stock solution (9 mg/ml) in DMSO. The pB6 phosphopeptide\textsuperscript{36} was labeled according to the same scheme and was used for controls. The mixtures were wrapped with aluminum foil and incubated overnight at room temperature, then the reaction was quenched by the addition of Tris to a final concentration of 1 M. The FITC-labeled peptides were separated from the unreacted label by SEC on a 2 ml Sephadex G10 column equilibrated and run using a 20 mM Tris-HCl buffer, pH 7.5,
150 mM NaCl at a 0.5 ml/min flow rate. The runs were operated by the Prostar 335 system (Varian Inc., Australia) following the full-spectrum absorbance. The resulting spectrum, and absorbance at 280 (A_{280}) and 493 nm (A_{493}) in particular, was used to estimate the phosphopeptide concentration (C_{peptide}), taking into account FITC absorbance at 280 nm using the formula \[ C_{peptide} = (A_{280} - 0.3 \times A_{493}) / I_{peptide, 280} \]

where \( I_{peptide, 280} \) is the extinction coefficient of the peptide at 280 nm (for all peptides in the study 5500 M^{-1} cm^{-1}). FITC concentration was determined from the FITC-specific absorbance at 493 nm using the extinction coefficient of 68,000 M^{-1} cm^{-1}. The completion of the labeling reaction was confirmed by LC-MS on a Bruker Impact II mass-spectrometer (Supplementary Table 4 and Supplementary Figure 8). The labeling efficiency was 74–76% for all phosphopeptides. Labeled peptides were stored in aliquots at −80 °C.

**Plasmids and mutagenesis**

The SARS-CoV-2 N sequence (Uniprot ID: P0DTCD9) was cloned as described previously.26 The protein contained a N-terminal His\(\varepsilon\)tag cleavable by rhinovirus 3C protease, which left extra residues GPA on the N terminus. The wild-type SARS-CoV-2 N plasmid was used as template to PCR amplify the S197L mutant using the S197L reverse mutagenic primer (5'-CTGGGAGTTAA ATTTCATGGACTGAGCTA-3'). For site-specific translational incorporation of phosphoserine or its non-hydrolyzable analog phosphono-methyl-alanine (nhpSer) at positions 197 or 205 of SARS-CoV-2 N using amber codon suppression in autonomous E. coli systems,27–30 we created the 197TAG and 205TAG mutants using the reverse mutagenic primers (5'-CTGGAGGTAA ATTTCATGGACTGAGCTA-3' for 197TAG or 5'-CAGG AGACTATCCCTACTGCTG-3' for 205TAG, the mutated codons are underlined) and DNA template being the SARS-CoV-2 N plasmid with a C-terminal His\(\varepsilon\)tag cleavable by 3C protease. The resulting PCR products including the C-terminal cleavable His\(\varepsilon\)tag were treated with NdeI/HindIII restriction endonucleases and then cloned into a pET21 vector (ampicillin resistance) in E. coli BL21(DE3) by cleavage of the His\(\varepsilon\)tag and cloned into the pET28 vector (kanamycin resistance) have been described previously.26 Homotypic constructs of the full-length human 14-3-3 protein isoforms beta (β), gamma (γ), eta (η), tau (τ), zeta (ζ), epsilon (ϵ), and sigma (σ), fused to the C terminus of the maltose-binding protein (MBP),28 were kindly shared by the laboratory of Prof. Gilles Trave (Université de Strasbourg, Illkirch, France). Parameters of proteins used are provided in Supplementary Table 5.

**Protein expression and purification**

Proteins were expressed in E.coli BL21(DE3) by the addition of IPTG up to 0.5 mM for 20 h at 25 °C (N proteins), or for 4–6 h at 37 °C (14-3-3 proteins). To ensure phosphorylation of SARS-CoV-2 N, its S197L mutant, or SARS-CoV N in E.coli cells, target proteins were co-expressed with the catalytically active subunit of mouse protein kinase A (PKA) encoded in a separate pACYC-PKA plasmid (chloramphenicol resistance), essentially as described earlier.26 The site-specifically phosphorylated SARS-CoV-2 N protein pS197 was expressed in BL21(DE3) ΔserB cells tailored to co-translational phosphoserine incorporation.50 This yielded partially phosphorylated purified protein, therefore we also produced the nhpSer197 protein based on the same plasmid containing a TAG197 (ampicillin resistance) in BL21(DE3) ΔserC cells specifically engineered to autonomously biosynthesize nhpSer from the central metabolite phosphoenolpyruvate.32 It was reported that phosphonomethylalanine (nhpSer) effectively imitates phosphoserine in previous 14-3-3 binding studies.32,33 Likewise, we also produced the SARS-CoV-2 N protein containing only nhpSer205 mimicking site-specifically phosphorylated protein at position 205. Given that this position within the RGT3226SPAR motif can be occupied by a serine in natural SARS-CoV-2 N variants (e.g., GenBank accession numbers, UDA99618.1, QWC76623.1, QRGZ7797.1, QX30013.1, QQN91819.1) and in related orthologs, e.g. in Bat-CoV HKU3 N,35 the use of nhpS205 version instead of pT205 in SARS-CoV-2 N was fully justified.

All N proteins, and Clu1 and Clu3 mutants of 14-3-3 used for crystallization, were purified by subtractive immobilized metal affinity and size-exclusion chromatography, where two IMAC steps were separated by 3C cleavage of the His\(\varepsilon\)tag.36 To remove large amounts of contaminating nucleic acids non-specifically bound to N, we used a contin-
uous washing step (50 column volumes) by 3 M NaCl before elution with imidazole during IMAC1. Most proteins were obtained at amounts exceeding 10 mg per 1 liter of bacterial culture. While the site-specifically modified SARS-CoV-2 N 197TAG and 205TAG were obtained at rather low yields (∼1–2 mg per 1 liter of bacterial culture) and were contaminated by truncated side-products, the resulting proteins were modified as expected, according to Phos-tag gels (10% polyacrylamide gels with 50 μM Mn²⁺ Phos-tag (Wako)). Despite SARS-CoV-2 N pS197 was purified in the presence of inhibitors of proteases (EDTA-free Pierce™ Protease Inhibitor Mini Tablets, cat. no. A32955) and phosphatases (orthovanadate, glycerophosphate and NaF), the product was considerably dephosphorylated. This prompted us to obtain also the nhpS197 and nhpS205 versions of this protein that resist dephosphorylation. SARS-CoV N was expressed and nhpS205 versions of this protein that resist dephosphorylation. SARS-CoV N was expressed and purified by a combination of the MBP-14-3-3 constructs was the same, but these proteins were purified using a combination of MBP-Trap column and size-exclusion chromatography.蛋白纯度在上海科技大学检测时，所需的凝胶条件为10%聚丙烯酰胺凝胶（Phos-tag）,便于对凝胶进行质谱分析。在特定情况下，Superdex 200 Increase 5/150柱是用0.45 ml/min的流量进行操作的。

**Analytical size-exclusion chromatography**

The interaction of N proteins and 14-3-3γ was analyzed using a Superdex 200 Increase 5/150 column (GE Healthcare) operated at a 0.45 ml/min flow rate on a Prostar 335 chromatographic system (Varian, Australia). When required, fractions were collected and analyzed by SDS-PAGE. In specific cases, a Superdex 200 Increase 10/300 column and a 0.8 ml/min flow rate was used. The buffer, used to equilibrate the columns, contained 20 mM Tris-HCl pH 7.6, 3 mM NaCl and 200–300 mM NaCl. We noticed that free unphosphorylated SARS-CoV-2 N.S197L absorbed onto the column when equilibrated with < 220 mM NaCl. Therefore, the reported experiments contained at least this much salt in the SEC buffer. Experiments were done at least three times with qualitatively very similar results.

**Multi-angle light scattering (MALS)**

To determine absolute masses of MBP-14-3-3γ, 14-3-3γ and its complex with phosphorylated SARS-CoV-2 N.S197L, we used SEC coupled to MALS. To do this, a chromatographic system was connected to the miniDAWN detector (Wyatt Technology) calibrated relative to the scattering from toluene. The concentration signal was obtained from SEC traces recorded at 280 nm and extinction coefficients 1.38 (mg/ml)⁻¹ cm⁻¹ for MBP-14-3-3γ, 1.13 (mg/ml)⁻¹ cm⁻¹ for 14-3-3γ, and 1.02 (mg/ml)⁻¹ cm⁻¹ for the 14-3-3γ/pN.S197L complex. The Mₜₜ distributions were calculated in Astra 8.0 software (Wyatt Technology) using dn/dc = 0.185. Protein content in the peaks was additionally analyzed by SDS-PAGE.

**Fluorescence anisotropy**

Fluorescence anisotropy was measured at 25 °C on a CDR Microplate Reader (BMG Labtech, Offenburg, Germany) using a set of bandpass filters (482 ± 16 nm and 530 ± 40 nm), FITC labeled phosphopeptides (100 nM, on a 20 mM HEPES-NaOH buffer, pH 7.5, 150 mM NaCl, 0.02% Tween 20) and 384-well plates (Black Nunc™ 384-Shallow Well Standard Height Polypropylene Storage Microplates, Thermo Fisher Scientific, cat. no. 267460). A series of concentrations of MBP-14-3-3 proteins in a 20 mM HEPES-NaOH buffer, pH 7.5, 150 mM NaCl, 0.01% Tween 20 (33 µl each) was diluted 2-fold by a 100 mM solution of either FITC-labeled peptide to obtain 66 µl samples containing 50 nM peptide, MBP-14-3-3 (from 0 to 110 µM) and 0.01% Tween 20. After 5 min incubation at room temperature, the mixture was diluted into 100 µl of the HEPES-NaOH buffer and fluorescence anisotropy was measured. The anisotropy was normalized to the baseline anisotropy of the buffer. The concentration dependence of the anisotropy was fitted to a binding model using Origin (OriginLab, Northampton, MA). The binding constant was determined as the dissociation constant, Kd.
temperature the fluorescence anisotropy readout did not change with time, indicating equilibrium. Then, each sample was split into three 20 μl aliquots, the plates were centrifuged for 1 min at 25 °C at 250 g to remove air bubbles, and the fluorescence anisotropy data were recorded. The triplicate measurements were converted into mean ± standard deviation values, which were used for the data presentation and fitting. To determine $K_D$ values, the binding curves were approximated using the quadratic equation in Origin 9.0 (OriginLab Corporation, Northampton, MA, USA). Selectivity coefficient reflecting the preferred 14-3-3 binding to the pT205 peptide than the pS197 peptide, which by definition is an equilibrium constant ($K_{eq} = 1/K_D$) for the corresponding displacement reaction, was determined as the ratio of $K_D$ for the pS197 peptide to $K_D$ for the pT205 peptide. To determine the correlation between selectivity or $-\log K_D$ values for the 14-3-3/phosphopeptide pairs and average ΔΔG values characterizing the affinity hierarchy reported earlier, we used the standard Pearson’s correlation coefficient $r$ and coefficient of determination $R^2$.

Crystallization and X-ray data collection

Crystallization was performed using 14-3-3σ surface entropy reduction mutants Clu1 or Clu3 (storage buffer 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 2 mM MgCl$_2$) mixed with either pS197 or pT205 phosphopeptide at a 1.5 protein:peptide molar ratio with the final protein concentration of 11.5 mg/ml. The best diffracting crystal for the 14-3-3σ Clu1/pT205 complex grew in condition H7 (0.2 M AmSO$_4$, 100 mM BisTris propane pH 7.5, 20% PEG 3350) of PACT screen (Qiagen, Hilden, Germany). Crystals were mounted in nylon loops and average $R_{	ext{merge}}$ = 0.057 to get a SAXS curve corresponding to the peak of the protein complex. This SAXS curve was further analyzed using ATSAS 2.8 program package.\footnote{Because of the tailing peak of the complex, the peak of free 14-3-3σ became contaminated and was not analyzed. To perform structural modeling of the complex, we took available crystallographic data for the SARS-CoV-2 N NTD monomer (resides 48–175, PDB ID: 7CDZ), CTD dimer (residues 254–364, PDB ID: 6WZO) and also considered as rigid body the well-documented hydrophobic $\alpha$1-helix within the interdomain linker (residues 220–232, PDB ID: 7PKU).\footnote{In our 14-3-3σ dimer structure with two SARS-CoV-2 N pT205 peptides (resides 203–209), we replaced 14-3-3σ by the 14-3-3γ dimer (resides 1–235, PDB ID: 6A5S). Considering rigid bodies above, we supplemented components of the assembly with missing N-terminal, C-terminal and linker residues absent from their atomistic structures (totaling ~26% of all 1338 residues in the complex), and systematically changed relative positions of two NTD protomers, two $\alpha$1 helices and CTD dimer relative to the 2:2 14-3-3σ/pT205 peptide complex using CORAL\footnote{To minimize the discrepancy between the calculated scattering profile and the experimental data. This fitting procedure showed high convergence ($\chi^2$ for all 20 models generated was close to 1). Alternative situations, represented by individual 14-3-3σ dimer or SARS-CoV-2 N dimer with supplemented flexible residues could not describe SAXS data for the complex ($\chi^2 > 200$ and 4.5, respectively)}.}

SEC coupled with small-angle X-ray scattering (SAXS)

SAXS data ($I(s)$ versus $s$, where $s = 4\pi\sin(\theta)/\lambda, 2\theta$ is the scattering angle and $\lambda = 0.96787$ Å) from the mixture of phosphorylated SARS-CoV-2 N S197L mutant with a 1.7x molar excess of 14-3-3γ (45 μl, 10 mg/ml), loaded onto a Superdex 200 Increase 32/300 column (Cytiva) and eluted at a 0.075 ml/min flow rate, were measured at the BM29 beam line (ESRF, Grenoble, France) using a Pilatus 2 M detector (data collection rate 0.5 frame/s; experiment session data DOI https://doi.org/10.15151/ESRF-ES-642726753). The running buffer contained 20 mM Tris-HCl, pH 7.6, and 150 mM NaCl. One thousand 1D SAXS frames acquired along the SEC profile were analyzed using CHROMIXS to get a SAXS curve corresponding to the peak of the protein complex. This SAXS curve was further analyzed using ATSAS 2.8 program package.\footnote{Because of the tailing peak of the complex, the peak of free 14-3-3σ became contaminated and was not analyzed. To perform structural modeling of the complex, we took available crystallographic data for the SARS-CoV-2 N NTD monomer (resides 48–175, PDB ID: 7CDZ), CTD dimer (residues 254–364, PDB ID: 6WZO) and also considered as rigid body the well-documented hydrophobic $\alpha$1-helix within the interdomain linker (residues 220–232, PDB ID: 7PKU).\footnote{In our 14-3-3σ dimer structure with two SARS-CoV-2 N pT205 peptides (resides 203–209), we replaced 14-3-3σ by the 14-3-3γ dimer (resides 1–235, PDB ID: 6A5S). Considering rigid bodies above, we supplemented components of the assembly with missing N-terminal, C-terminal and linker residues absent from their atomistic structures (totaling ~26% of all 1338 residues in the complex), and systematically changed relative positions of two NTD protomers, two $\alpha$1 helices and CTD dimer relative to the 2:2 14-3-3σ/pT205 peptide complex using CORAL\footnote{To minimize the discrepancy between the calculated scattering profile and the experimental data. This fitting procedure showed high convergence ($\chi^2$ for all 20 models generated was close to 1). Alternative situations, represented by individual 14-3-3σ dimer or SARS-CoV-2 N dimer with supplemented flexible residues could not describe SAXS data for the complex ($\chi^2 > 200$ and 4.5, respectively).}}

Crystal structure determination and refinement

X-ray structures were determined using MolRep\footnote{using an unliganded 14-3-3σ dimer (PDB ID: 5LU2) as a search model. Peptide residues were manually built into the difference electron density maps using Coot.\footnote{Structures were refined by Buster 2.10.4\footnote{Structures were refined by Buster 2.10.4 imposing NCS restraints and TLS and all-atom individual isotropic B-factor restrained refinement. The refined structures were validated using the validation algorithm in Phenix 1.10.\footnote{Structural illustrations were prepared using PyMOL 2.20 (Schrodinger, Inc.).}} imposing NCS restraints and TLS and all-atom individual isotropic B-factor restrained refinement. The refined structures were validated using the validation algorithm in Phenix 1.10.} imposing NCS restraints and TLS and all-atom individual isotropic B-factor restrained refinement. The refined structures were validated using the validation algorithm in Phenix 1.10.\footnote{Structural illustrations were prepared using PyMOL 2.20 (Schrodinger, Inc.).}

Accession numbers

PDB: 7QIK, 7QIP.

CRediT authorship contribution statement

Kristina V. Tugaeva: Investigation, Methodology, Visualization. Andrey A. Sysoev: Investigation. Anna A. Kapitonova: Investigation.
DATA AVAILABILITY
Data will be made available on request.

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Declaration of competing interests

The authors declare no conflicts of interest.

Author Contributions

NNS and AAA conceived studies; PZ and RBC – prepared pSer and nhpSer incorporating cells; KVT, AAS, AAK, JLRS, NNS – expressed and purified proteins; KVT – performed fluorescence anisotropy experiments; JLRS – crystallized proteins; NNS – determined crystal structures and did SAXS modeling; KVT, RBC, AAA, NNS – analyzed data; NNS and KVT made illustrations; NNS wrote the paper with input from RBC and AAA.

Data and materials availability

The refined models and structure factors have been deposited in the Protein Data Bank under the accession codes 7QIK and 7QIP. All materials are available from the corresponding authors upon reasonable request.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2022.167891.

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Keywords:
host-virus interactions;
RNA viruses;
14-3-3 protein;
phosphopeptide binding;
binding selectivity

Abbreviations:
CORAL, COmplexes with RAndom Loops; CTD, C-terminal domain; DMSO, Dimethyl sulfoxide; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; FITC, Fluorescein 5-isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSPB6, Small heat shock protein B6; IDR, Intrinsically disordered region; IMAC, Immobilized metal affinity chromatography; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LC-MS, Liquid chromatography-mass spectrometry; MALS, Multi-Angle Light Scattering; MBP, Maltose-binding protein; MERS, Middle East Respiratory Syndrome; NCS, Noncrystallographic symmetry; NTD, N-terminal domain; PEG, Polyethylene glycol; PKA, Protein kinase A; SARS, Severe Acute Respiratory Syndrome; SAXS, Small-angle X-ray scattering; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; SEC, Size-exclusion chromatography; SR region, Serine/arginine-rich region; TLS, Translation/Libration/Screw

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