Synergy of aromatic residues and phosphoserines within the intrinsically disordered DNA-binding inhibitory elements of the Ets-1 transcription factor

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The E26 transformation-specific (Ets-1) transcription factor is autoinhibited by a conformationally disordered serine-rich region (SRR) that transiently interacts with its DNA-binding ETS domain. In response to calcium signaling, autoinhibition is reinforced by calmodulin-dependent kinase II phosphorylation of serines within the SRR. Using mutagenesis and quantitative DNA-binding measurements, we demonstrate that phosphorylation-enhanced autoinhibition requires the presence of phenylalanine or tyrosine (Φ) residues adjacent to the SRR phosphoaceptor serines. The introduction of additional phosphorylated Ser-Φ-Asp, but not Ser-Ala-Asp, repeats within the SRR dramatically reinforces autoinhibition. NMR spectroscopic studies of phosphorylated and mutated SRR variants, both within their native context and as separate trans-acting peptides, confirmed that the aromatic residues and phosphoserines contribute to the formation of a dynamic complex with the ETS domain. Complementary NMR studies also identified the SRR-interacting surface of the ETS domain, which encompasses its positively charged DNA-recognition interface and an adjacent region of neutral polar and nonpolar residues. Collectively, these studies highlight the role of aromatic residues and their synergy with phosphoserines in an intrinsically disordered regulatory sequence that integrates cellular signaling and gene expression.

Intrinsically disordered protein regions (IDRs) are increasingly recognized for their prevalence in the eukaryotic proteome and their roles in normal biological processes, as well as in disease (1–3). IDRs serve as flexible linker sequences between modular domains and as key components of complex protein-interaction networks. These sequences are often sites of posttranslational modifications, and their plasticity enables accessible and reversible interactions necessary for the integration of cellular signals.

The autoinhibition of the E26 transformation-specific (Ets-1) transcription factor provides an illuminating example of how a flexible, disordered region can modulate the regulatable DNA-binding ETS domain and thereby tune it for biological control by calcium-dependent phosphorylation and cooperative protein partners (4). The inhibitory module (IM) of Ets-1 is composed of four α-helices (HI-1, HI-2, HI, and HS) that pack onto the ETS domain distal from the DNA interface (Fig. 1A) (5). Helix HI-1 is marginally stable and unfolds on DNA binding, thus implicating an allosteric mechanism of inhibition (6, 7). The modest twofold repression afforded by the IM is increased to ~20-fold by an intrinsically disordered serine-rich region (SRR) (8, 9). The dynamic SRR interacts transiently with both the ETS domain DNA-binding interface and the IM, and thus plays steric and allosteric inhibitory roles (10). Phosphorylation of five SRR serines by calmodulin-dependent kinase II (CaMKII) leads to a dramatic ~500-fold autoinhibition (11). In parallel, the conformational flexibility of the IM, and the ETS domain decreases (9, 10). By analogy to the well-characterized lac repressor (12), this flexibility is hypothesized to play a central role in DNA binding.

This study interrogates the physicochemical basis of the transient interactions underpinning Ets-1 autoinhibition. Through mutational analyses, we found that Phe/Tyr (Φ) residues in the SRR act synergistically with nearby phosphoserines to reinforce autoinhibition. Complementing these biochemical assays, we used NMR spectroscopy to investigate the intermolecular interactions of peptides, corresponding to the SRR, with the IM and ETS domain. The trans-SRR peptides retained inhibitory activity and formed dynamic complexes, lacking any persistent induced conformation, via the same interface detected “in cis” by chemical shift perturbation (CSP) and paramagnetic relaxation enhancement (PRE) experiments. Intermolecular binding was also enhanced by phosphorylation and weakened on alanine substitution of the Phe/Tyr residues. These studies revealed the synergy between aromatic residues and phosphoserines in the function of an IDR.

Results
Aromatics Are Essential for Phosphorylation-Enhanced Autoinhibition. Sequence alignment of the SRR, which is unique to Ets-1 and Ets-2, revealed a repetitive pattern of Phe/Tyr and Asp/Glu residues adjacent to the CaMKII phosphoaceptors (Fig. 1B and Fig. S1). We tested the roles of these conserved residues

Significance

Eukaryotic proteins often contain intrinsically disordered regions that lack well-defined conformations, yet still play key roles in numerous biological processes. The molecular mechanisms underlying the functions of such unstructured regions are often poorly understood. In the case of the E26 transformation-specific transcription factor, DNA binding by the ETS domain is autoinhibited through transient interactions with an adjacent disordered serine-rich region (SRR). Phosphorylation of the SRR in response to cellular signals increases these interactions and thereby reinforces autoinhibition. In this study, we demonstrate that phosphorylation-enhanced autoinhibition requires the presence of phenylalanine or tyrosine residues neighboring the SRR phosphoaceptor serines. This highlights a previously unrecognized role of aromatic residues and their synergy with phosphoserines in an intrinsically disordered regulatory sequence.


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Intramolecular interactions of the SRR with the ETS domain are dependent on aromatic residues and phosphoserines. (A) Ets-1 schematic: PNT domain (cyan), SRR (yellow), IM (purple), ETS domain (red). ΔN279 with SRR residues, CaMKII phosphoacceptors S282 and S285 (†), and four mutated aromatic residues (4A; red, orange), and four mutated aromatic residues (4A; red, orange), and four mutated aromatic residues (4A; red, orange), and four mutated aromatic residues (4A; red, orange). (B) CSPs for main-chain amides and side-chain amides/indoles in ΔN279 (orange bars) and ΔN279^2P (black lines) on 4A substitutions. See Fig. 5A for other comparisons. (C) CSPs for residues 301–440 of ΔN279 on 4A mutation mapped on ΔN301 [Protein Data Bank (PDB) ID 1R36] with perturbed side chains in stick format (red: Δδ > 0.125 ppm, yellow: 0.125 ppm > Δδ > 0.08 ppm, gray: Δδ < 0.08 ppm or prolines). (D) Surface representation of ΔN301 showing the positively charged (blue, Arg, Lys), negatively charged (red, Asp, Glu), hydrophobic (green), and neutral polar (white) residues. Using ΔN279, an Ets-1 species with a shorter SRR that retains phosphorylation-dependent autoinhibition. Consistent with previous studies (9, 10), the truncated SRR caused 18-fold inhibition of DNA binding for ΔN279 relative to ΔN301 (only the IM and ETS domain), and phosphorylation of S282 and S285 increased this to 1,900-fold (Table 1). Serine substitution with glutamates or aspartates increased inhibition to only ~75-fold. The partial effects of these phosphomimetics may arise from charge (−2 vs. −1 at pH 8) and/or structural differences. Lowering the SRR net negative charge by substitution of D284, D287, E289, and D290 with alanines did not alter basal auto-inhibition. (CaMKII failed to phosphorylate these mutants, and thus effects on the reinforcement of autoinhibition could not be measured.) Replacing the negative charges of the added phosphates with positive charges via arginine substitutions at S282 and S285 also failed to reinforce autoinhibition. These findings indicated that the SRR does not act by a simple global electrostatic effect, but rather through a specific role of the phosphoserines.

Next, four conserved aromatic residues in the SRR were investigated (Table 1). Alanine (or glycine) substitutions reduced phosphorylation-dependent inhibition to only approximately threefold for ΔN279-4A compared to ΔN279-4A^2P with an ~100-fold effect for the corresponding WT species, while showing minimal effects on basal inhibition. Similar results were found in mutational tests of ΔN248 and native Ets-1, which have the full SRR with five phosphoacceptor serines. Substitution of the four aromatics in ΔN279 to valines had comparable effects to alanine replacements, strongly suggesting that the aromatic character of tyrosines and phenylalanines is essential for phosphorylation-dependent inhibition. The importance of phosphorylated serines and aromatic residues within a possible repeating unit was tested with SRR variants containing one, two, or four additional Ser-φ-Asp repeats (Table 2). Although there was only a minimal effect on basal inhibition, phosphorylation weakened DNA binding up to 10,000-fold. In contrast, Ser-Ala-Asp repeats lacking aromatics were ineffective even in the highly phosphorylated state. We conclude that the phosphoserines and aromatic residues contribute synergistically to autoinhibition.

**Aromatic Residues and Phosphorylation Mediate Intramolecular Interactions.** CSP and ^15^N relaxation measurements probed the conformation and intramolecular interactions of the 4A version of the ΔN279 SRR. A comparison of the ^15^N-heteronuclear single quantum correlation (HSQC) spectra of WT and the 4A mutant showed the largest CSPs were within the SRR (Fig. 1B). Importantly, amides within the ETS domain and IM also exhibited small CSPs. Phosphorylation of either WT or the 4A mutant also led to similar patterns of CSPs (Fig. S2A). However, these spectral perturbations were generally larger on phosphorylation of WT than the 4A mutant and on mutation of the phosphorylated rather than the nonphosphorylated protein. When mapped onto the ΔN301 structure, residues with the largest CSPs clustered near helix H3 and the following β-strand S4 of the ETS domain, as well as in the H1-2/H1 and S4/H4 loops (Fig. 1C). This surface is very similar to that detected by PRE measurements of WT ΔN279 with a nitroxide spin label covalently linked to Cys278 (Fig. S2B and C). These NMR approaches defined the aromatic- and phosphoserine-dependent interface, including positively charged residues near the DNA interface, as well as an adjacent patch of hydrophobic and neutral polar residues. Thus, both

**Table 1. Mutations demonstrate a critical role for SRR aromatics in phosphorylation-enhanced autoinhibition of ΔN279 DNA binding**

<table>
<thead>
<tr>
<th>Protein</th>
<th>SRR sequence*</th>
<th>Kp (x10^-11 M)†</th>
<th>Fold inhibition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔN301</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>2.5 ± 0.5</td>
<td>—</td>
</tr>
<tr>
<td>ΔN279</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>44 ± 4.4</td>
<td>18 ± 2.7</td>
</tr>
<tr>
<td>ΔN279</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>4,700 ± 580</td>
<td>1,900 ± 320</td>
</tr>
<tr>
<td>ΔN279</td>
<td>S-R</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>180 ± 50</td>
</tr>
<tr>
<td>ΔN279</td>
<td>S-D</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>190 ± 7.8</td>
</tr>
<tr>
<td>ΔN279</td>
<td>DE-A</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>71 ± 16</td>
</tr>
<tr>
<td>ΔN279</td>
<td>S-R</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>45 ± 4.9</td>
</tr>
<tr>
<td>ΔN279</td>
<td>4G</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>44 ± 8.7</td>
</tr>
<tr>
<td>ΔN279</td>
<td>4G</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>130 ± 11</td>
</tr>
<tr>
<td>ΔN279</td>
<td>4A</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>44 ± 1.7</td>
</tr>
<tr>
<td>ΔN279</td>
<td>4A</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>ΔN279</td>
<td>4V</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>ΔN279</td>
<td>4V</td>
<td>RVP A YDS F D Y D Y E D Y P A A L P N H K P</td>
<td>160 ± 33</td>
</tr>
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*Phosphoserines (S) and mutated/modified sites are bold underlined.
†Measured by EMSA for a consensus Ets-1 site. Inhibition relative to ΔN301.
Table 2. Additional Ser-φ-Asp repeats mediate higher levels of phosphorylation-dependent autoinhibition of ΔN279 DNA binding

<table>
<thead>
<tr>
<th>Protein</th>
<th>SRR sequence*</th>
<th>$K_D$ (×10^{-11} M)$^\dagger$</th>
<th>Fold inhibition$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔN279$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y ED-Y PAALPNHKPSRR A**</td>
<td>44 ± 4.4</td>
<td>18 ± 2.7</td>
</tr>
<tr>
<td>ΔN279$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR B**</td>
<td>4,700 ± 500</td>
<td>1,900 ± 320</td>
</tr>
<tr>
<td>3-unit SRR$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR C</td>
<td>44 ± 3.8</td>
<td>18 ± 2.2</td>
</tr>
<tr>
<td>4-unit SRR$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR D</td>
<td>5,900 ± 2,700</td>
<td>2,400 ± 1,200</td>
</tr>
<tr>
<td>5-unit SRR$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR E</td>
<td>33,000 ± 17,000</td>
<td>13,000 ± 5,700</td>
</tr>
<tr>
<td>6-unit SRR$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR F</td>
<td>59 ± 1.7</td>
<td>23 ± 7.0</td>
</tr>
<tr>
<td>6-unit SRR$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR G</td>
<td>&gt; 200,000</td>
<td>&gt; 100,000</td>
</tr>
<tr>
<td>4-unit 4A$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR H</td>
<td>40 ± 4.4</td>
<td>16 ± 2.6</td>
</tr>
<tr>
<td>4-unit 4A$^{\phi P}$</td>
<td>RVPS-YDSDFD-Y YD-Y PAALPNHKPSRR I</td>
<td>210 ± 46</td>
<td>84 ± 21</td>
</tr>
</tbody>
</table>

*Constructs for tandem repeats, delineated by hyphenation, retained WT SRR length by replacing 3, 6, or 12 amino acids. Phosphoserines (SP) and mutated/modified sites are bold underlined.

†Measured by EMSA for a consensus Ets-1 site. Fold inhibition relative to ΔN301 (Table 1).

electrostatic and hydrophobic/van der Waals forces contribute to the SRR-ETS domain interactions (Fig. 1D).

The fast subnanosecond timescale backbone motions of the mutant SRR-4A in the context of ΔN279 were also investigated by 1H(15N)-nuclear Overhauser effect (NOE) measurements (Fig. S3). In both the unmodified and phosphorylated forms, the SRR-4A residues showed significantly lower amide NOE values, and hence greater mobility, than residues in the ordered regions of the protein. However, the NOE values of residues throughout the SRR-4A increased on phosphorylation. Consistent with a previous 15N relaxation analysis of the WT ΔN279 (10), we conclude that the SRR functions through intramolecular interactions with the ETS domain and inhibitory elements and that its dynamic character scales inversely with its phosphorylation-dependent inhibitory activity.

**Trans-SRR System Recapitulates Autoinhibition.** We developed a trans system wherein ΔN279 is represented by a SRR peptide and ΔN301 (Fig. 2A). The intermolecular system opened the door to new concentration-dependent structural studies of the SRR. Addition of a peptide version of the phosphorylated SRR$^{2P}$ weakened binding of ΔN301 to DNA (Fig. S4), whereas peptides that either lacked phosphoserines, or had the 4φA mutations, failed to measurably inhibit in trans. In NMR-monitored titrations (Table 3, Fig. S5A, and Fig. S5B), the SRR$^{10}$ peptide showed the highest affinity for ΔN301 with an equilibrium dissociation constant ($K_D$) of 100 μM. The absence of the two phosphates in SRR$^{10}$ caused a 16-fold affinity reduction ($K_D$ ~ 1,650 μM). Increasing ionic strength from 50 to 200 mM NaCl reduced the affinity of both SRR$^{10}$ and SRR$^{2P}$ peptides for ΔN301 by a modest two- to threefold, indicating an electrostatic component to binding. Replacing the four aromatic residues in the SRR peptide with alanines (SRR-4φA$^{10}$) severely weakened binding, precluding reliable $K_D$ measurement. Phosphorylation of the mutant SRR-4φA$^{10}$ peptide partially restored its affinity for ΔN301 ($K_D$ ~ 260 μM). Thus, the SRR peptides recapitulated the cis-acting inhibitory properties of ΔN279.

To identify the residues of the SRR that interact with ΔN301, we titrated the WT 13C/15N-labeled peptides with unlabeled protein (Fig. 3A and B and Fig. S6). The amides of D284, S285, F286, Y288, and E289, as well as the aromatic nuclei of F286, exhibited chemical shift changes on addition of ΔN301 to SRR$^{10}$. In the case of the SRR$^{2P}$ peptide, backbone amide and side-chain aromatic nuclei of residues 283–291 inclusive showed larger CSPs, likely due to its higher affinity and hence increased saturation levels. Together, these titrations confirmed that trans binding to ΔN301 involves both phosphoserine and aromatic moieties within the SRR peptides.

We also used reciprocal NMR-monitored titrations to determine and compare the interaction surface of the four SRR peptides on ΔN301. Addition of each to the 15N-labeled protein led to similar patterns of CSPs, albeit with varying magnitudes due, at least in part to differing affinities that precluded the formation of the fully saturated complexes (Fig. 4 and Fig. S5 B and C). Residues of ΔN301 showing the largest CSPs clustered within the loop between helices H1-2 and H1 linking the IM to the ETS domain, as well as within H3 and strand S3 of the ETS domain. On more detailed inspection, amides near the C terminus of H3 and in the loop to S3 (including W338, L341, and K399) showed colinear CSPs when titrated with the four peptides (Fig. 4). These spectral changes might arise from common local conformational perturbations linked to autoinhibition. A similar phenomenon of colinear CSPs paralleling DNA-binding affinity is seen with cis-acting SRR variants (9). In contrast, for
other residues (including L337, W375, and Y395) clustering near the N terminus of H3 and the H2-H3 turn, the CSP patterns differed for SRR-4A  
and SRR-4A relative to SRR and SRR-3. These differences might reflect CSPs due to aromatic ring currents, thus suggesting that the SRR aromatic residues localize to this region of ΔN301. Overall, the same surface regions were identified for the intramolecular interactions within ΔN279 by PRE and CSP studies (Figs. S2 and S7). Thus, the interactions of the trans-peptides recapitulate the intramolecular allosteric and steric mechanisms of SRR-mediated autoinhibition.

**Discussion**

Aromatic Residues in the SRR Are Critical for Phosphorylation-Dependent Autoinhibition. Previous studies demonstrated that the intrinsically disordered SRR is required for both basal and phosphorylation-enhanced autoinhibition of Ets-1 DNA binding, yet the underlying molecular interactions were largely unknown. IDRAs are generally depleted in hydrophobic amino acids (16), and thus it is striking that the CaMKII phosphoacceptor serines contributing to autoinhibition are adjacent to Phe/Tyr (φ) residues within a Ser-φ/Asp/Glu repeat. There are two repeats in the truncated SRR (282SYD-SFD-287) and a third in the full SRR (251SFE-253) (9, 11). Mutation of these two aromatic residues and two additional ones within the truncated SRR to glycines, alanines, or valines strongly reduced phosphorylation-enhanced autoinhibition. Conversely, the presence of additional Ser-φ/Asp repeats dramatically increased the effect, whereas even six phosphorylated Ser-Ala-Asp repeats did not reinforce autoinhibition. The contribution of the aromatic residues was confirmed using the trans-SRR system. On addition of ΔN301, NMR signals from the aromatic residues in the SRR peptides shifted, indicating that they are involved directly in the intramolecular interface. Furthermore, the SRR-4A peptide bound ΔN301 with approximately three-fold lower affinity than did the SRR peptide, and the very weak interaction of the SRR-4A peptide could not be quantified. Thus, both phosphoserines and adjacent Phe/Tyr residues within the SRR act synergistically to inhibit Ets-1 DNA binding.

**Fuzzy Interactions Between the SRR and ETS Domain.** The interactions between the SRR and ETS domain can be described as fuzzy based on their transient nature and the lack of any induced, persistent structure (17). This conclusion, which stands in sharp contrast to examples of disordered regions that fold into well-defined structures on partner binding, derives from several lines of evidence. Whether linked to the IM and ETS domain as in ΔN279 or as an isolated peptide, the SRR residues have random coil chemical shifts. Furthermore, neither phosphorylation nor ΔN301 binding causes any chemical shift changes indicative of a predominant induced structure. Amide 1H(15N)-NOE experiments also demonstrated that the residues in the SRR are significantly more flexible on a subnanosecond timescale than those in the well-ordered ETS domain. However, the backbone motions of the SRR are not unrestricted. Based on the substantially lower NOE values of the SRR-4A peptide relative to the WT peptide, this might result from hydrophobic clustering of the four aromatic residues. The motions of the SRR also dampen with increased binding to the ETS domain on phosphorylation. Thus, the SRR appears to rapidly interconvert between an ensemble of conformations that are partially restrained by transient interactions, both within the SRR itself and with the ETS domain. This ensemble may involve binding of the flexible SRR at one predominant site or at multiple sites on the ETS domain. These two possibilities were not resolved due to coarse nature of CSP and PRE data and the absence of detectable interproton NOEs between the SRR and ETS domain (10).

The interaction surface of Ets-1 contacted by the SRR helps us understand how the dynamic complex functions in autoinhibition. This surface, which spans from the ETS domain to the IM, was broadly defined through NMR-monitored titrations of ΔN301 with the trans-SRR peptides, PREs from an N-terminal MTSL spin label on ΔN279, and CSPs of ΔN279 resulting from 4φA-mutation and phosphorylation of the SRR. Overall, this region has a net positive charge due to several Arg and Lys residues, whereas the SRR of ΔN279 contains four Asp and Glu residues, as well as two phosphoacceptor serines. Thus,
electrostatic forces likely contribute to the interaction of the SRR with the ETS domain and IM. This conclusion is further supported by the modestly weakened $K_D$ values of the SRR peptides for $\Delta N301$ with increasing ionic strength. However, glutamate and aspartate are only partial mimics of phosphoserine, and removing the four carboxylates or introducing two arginines does not impair basal autoinhibition. Therefore, more than simple Coulombic interactions are at play. Indeed, the SRR-interacting surface of the Ets-1 ETS domain overlaps with a relatively large patch of neutral polar and nonpolar residues between the IM and DNA-recognition helix (Fig. 1D). With partially exposed side chains, Tyr307 (H1-1), Tyr329 (H1-2), Trp338 (H1), Tyr395 (H3), and Tyr396 (H3) are included in this patch. Thus, hydrophobic and van der Waals interactions involving aromatic residues could also help localize the SRR to this region of Ets-1.

**Role of Aromatic Residues in IDRs.** The observation that phosphorylation-enhanced autoinhibition requires the presence of aromatic residues suggests that the SRR functions through more than just a simple collection of weak electrostatic and hydrophobic/van der Waals contacts. What then are the underlying forces by which aromatic residues integrate with phosphoserines to mediate SRR-ETS domain binding? One possible mechanism could involve the simultaneous association of adjacent phosphoserines and aromatic residues with lysine and arginine side-chains in the DNA-recognition interface of Ets-1 via ion-pair and cation interactions, respectively (18, 19). This would result in cooperative, multivalent binding of the SRR with the ETS domain. Alternatively, the negatively charged phosphates could interact with solvent and help drive a hydrophobic clustering of the neighboring aromatic residues either within the SRR or with the hydrophobic residues on the surface of the ETS domain and the IM. In support of this hypothesis, as a “salting-out” anion in the Hofmeister series, phosphate decreases the solubility of nonpolar solutes and stabilizes proteins, possibly through the ordering of water (20–22).

Functionally critical aromatic groups in IDRs are also found in the disordered activation domain (EAD) of the transcription factor EWS/FLI. This ~280 residue region is a low complexity sequence comprised mainly of a repeating SYGQQS motif. The activity of the EAD in transcriptional regulation and oncogenesis is strongly dependent on the presence of multiple tyrosines within these repeats (23, 24). A structural contribution of the multiple tyrosines in the (G/S)Y(G/S) repeats of all related FET proteins (FUS, EWS, and TAF15) is also suggested by the reversible polymerization of these IDRs (25). This may mediate biological regulation because the tyrosines are essential for phosphorylation-regulated binding of the EAD to the C-terminal domain (CTD) of RNA polymerase II. Although the EAD tyrosines are hypothesized to form fuzzy polycation/π interactions with yet unidentified positively charged partner proteins (26), the molecular forces driving these processes, which show both similarities and differences to the autoinhibitory SRR of Ets-1, remain to be established.

**ETS Factor Autoinhibition.** The Ets-1 autoinhibitory mechanism has both common and distinct features relative to those exploited by other ETS transcription factors and thus provides a route to specificity within this group of proteins that otherwise share multiple tyrosines in the (G/S)Y(G/S) repeats of all related FET proteins.

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**Fig. 3.** Characterizing the trans-SRR peptide structure, dynamics and $\Delta N301$-binding interface. (A) Overlaid $^{15}$N-HSQC and $^{13}$C-HSQC spectra of $^{15}$Clabeled SRR peptides in the indicated protein-peptide molar ratios. pS285* is from a population of peptide with a cis Val280-Pro281 bond. See Fig. S6 for full spectra. (B) Amide CSPs observed upon addition of $\Delta N301$ to the labeled SRR0P (~25% saturation; blue bars) and SRR2P (~85% saturation; red line). Peptide interface

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Desjardins et al. | PNAS | July 29, 2014 | vol. 111 | no. 30 | 11023
SRR-4 exhibited a pattern of increasing shift perturbations in the order SRR-37%; blue), or SRR2P (64%), or SRR0P (46%, or SRR1P (1.1 mM, 78%; purple), SRR0P (1.0 mM, 37%; blue), or SRR2P (640 μM, 84%; red). Residues with large (−0.1 ppm; red) and medium (0.07−0.1 ppm; yellow) amide or iodide CPSs on titration with SRR2P are mapped on ΔN301 (PDB ID 1R36). Perturbed side chains in stick format. Other peptides were similar (Fig. S5 B and C). Residues (Left) exhibited a pattern of increasing shift perturbations in the order SRR-44ΔP < SRR-37ΔP < SRR0ΔP < SRR2ΔP, suggestive of similar structural perturbations. Residues (Right) showed peptide-specific changes.

highly related DNA-binding properties (4). Ets-related gene (ERG) is regulated by a dynamic N-terminal sequence that perturbs its DNA-binding helix in a manner akin to that of the Ets-1 SRR (27). However, this sequence is almost devoid of aromatic residues. Furthermore, ERG lacks an equivalent IM and is not known to be modulated at the level of DNA binding by phosphorylation. In contrast, ETV6 does not contain an equivalent SRR and instead is autoinhibited by a C-terminal helix that sterically blocks its ETS domain interface and unfolds upon binding both specific and nonspecific DNA sequences (28). In all of these cases, additional DNA binding proteins can counteract or bypass the negative effects of autoinhibition and thereby provide added specificity to each ETS factor.

Materials and Methods

The experimental procedures are provided in detail as SI Materials and Methods.

Sample Preparation. Unlabeled SRR peptides (N-terminal acetylated and C-terminal amidated) were synthesized with or without phosphoserine (Biomatik). Isootopically labeled peptides were expressed in Escherichia coli and isolated as glutathione S-transferase- and His-tagged fusions, followed by tag cleavage and reverse phase HPLC purification. Murine ΔN301 and ΔN279 variants were expressed in E. coli, isolated by multiple chromatography steps (9, 10), and phosphorylated in vivo with CaMII kinase (9).

EMSAs. DNA-binding affinity of each Ets-1 variant was measured as previously described (9) with labeled duplexed 27-mer oligonucleotides: 5′-TCAGGCGCAAAGCGGAATGTAGTCGCT-3′ (top strand); GGAA consens (noted in bold) and 5′-TCAGGACACTCCTCCTCGTTGCGG-3′ (bottom strand). K0 values represent the average of at least two independent experiments ± SEM.

NMR Spectroscopy. Samples were in 20 mM Mes, 50 mM NaCl, 0.5 mM EDTA, 0.02% NaN3, and 5 mM DTT with 5–10% (vol/vol) D2O at pH 6.5. NMR data were recorded at 25 or 28 °C on 500- and 600-MHz spectrometers. Chemical shift perturbations were calculated from the combined 1H and 15N shift changes as Δ = (0.2 × ΔδH)2 + (ΔδN)2/2. Peptide-protein titrations were monitored via sensitivity-enhanced 15N-HSQC spectra, and K0 values determined by fitting Δδ to a 1:1 binding isotherm.

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Supporting Information

Desjardins et al. 10.1073/pnas.1401891111

SI Materials and Methods

Unlabeled Synthetic Serine-Rich Region Peptides. The following chemically synthesized peptides, corresponding to the truncated serine-rich region (SRR) of E26 transformation-specific (Ets-1) (residues 279–300) of murine Ets-1 were ligated into the SRR coding sequence for the SRR. The resulting parental vector was used for purification. The peptides were expressed in E. coli (DE3) cells. Cultures of 1 L were grown at 37 °C to OD600 = 0.9, with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown at 30 °C for 3 h in the presence of 35-methyl thymidine (35-M) and 1 mM DTT. After induction, the peptides were purified further by reversed-phase HPLC with a C-18 column. The peak fractions in the eluant were pooled, lyophilized, readjusted to pH 6.5, and dialyzed in Float-A-Lyzer G2 (SpectrumLab) for 48 h against the same buffer. Concentrations of SRRp and SRRP were determined by absorbance using a predesigned molar absorptivity $\varepsilon_{280} = 4,470 \text{ M}^{-2}\text{cm}^{-1}$. Amino acid analysis (Sick Children’s Hospital, Toronto, Canada) was used to quantify samples of SRR-4pA and SRR-4pA-P.

Protein Expression and Purification. The plasmids encoding residues 301–440 (denoted as SN301; Fig. 1) and N-terminal His$_6$-tagged 279–440 (SN301) of murine Ets-1 were obtained by ligating synthetic, codon-optimized genes (Blue Heron) into pET-28a vectors following modifications. To obtain phosphorylated peptides, phosphorylation was performed after the first round of Ni$^{2+}$ affinity chromatography, followed by purification on Mono-Q resin, as described below. The GST-His$_6$-tagged WT or WT SRR (residues 279–300) of murine Ets-1 were ligated into a pGEXW2T vector to tandem tag with glutathione S-transferase (GST) and His$_6$. The GST component of the tag increased solubility of the peptides, whereas the His$_6$ component was used for purification. The peptides were expressed in E. coli BL21 (DE3) and purified as described above for SN301 with the following modifications. To obtain phosphorylated peptides, phosphorylation was performed after the first round of Ni$^{2+}$ affinity chromatography, followed by purification on Mono-Q resin, as described below. The GST-His$_6$-tag was then cleaved with thrombin, and the products were rechromatographed on a Ni$^{2+}$ affinity chromatography to remove the cleaved tag and uncleaved product. The flow-through containing the peptides was further purified by reversed-phase HPLC with a C-18 column. The peak fractions in the eluant were pooled, lyophilized, readjusted to pH 6.5, and dialyzed

In Vitro Protein Phosphorylation by Calmodulin Kinase II. Protein and peptides were diluted to a final concentration of 25 μM in 50 mM Tris, 0.5 mM magnesium acetate, 2 mM DTT, and 10% (vol/vol) DMSO at pH 7.7. Calmodulin kinase II (CaMKII; 1 μM, expressed via a baculovirus in Sf9 cells and activated by preincubation at 30 °C for 10 min with 5 μM calmodulin, 0.5 mM CaCl$_2$ and 1 mM ATP) was added to the protein or peptide stock solutions. The solutions were mixed at 30 °C up to 16 h, yielding products with one or two phosphorylated sites as determined by ESI-MS or MALDI-ToF MS. After dialysis to 20 mM Tris, pH 8.3, 0.5 mM EDTA, 10% glycerol, and 1 mM TCEP, the products were purified as described below for SN301 with the following modifications. After cleavage of the Ni$^{2+}$ affinity column by thrombin, the products were eluted into NMR sample buffer with an Amicon filtration device (3-kDa MWCO). These sites were identified previously as Ser282 and Ser285 via MS sequencing and, in the case of isotope-labeled peptides, confirmed by NMR spectroscopy.

EMSAs. DNA-binding assays of Ets-1 proteins were performed using a $^{32}$P radio-labeled, duplexed 27-bp oligonucleotides containing a high affinity ETS binding site: 5′-TCGAGGCACTCAGCTGGTCTC-3′ (top strand) and 5′-TCGAGGCACTGCTGCTTTGCG-3′ (bottom strand). Boldface GGAA marks the consensus sequence motif for ETS family DNA binding proteins.
binding. The oligonucleotides were labeled with \([γ-32P]\) ATP using T4 polynucleotide kinase (Invitrogen) and annealed by boiling for 5 min and slowly cooling over 6–8 h. The DNA concentration was kept constant at 2.5 × 10^{-12} M, whereas the Ets-1 protein concentrations ranged over six orders of magnitude. The final binding reactions were incubated at 4 °C for 3–4 h in a buffer containing 25 mM Tris, pH 7.9, 0.1 mM EDTA, 60 mM KCl, 6 mM MgCl₂, 200 µg/ml BSA, 10 mM DTT, 2.5 ng/µL poly(dIdC), and 10% (vol/vol) glycerol at pH 8, and then resolved on an 8% (wt/vol) native polyacrylamide gel. Binding reactions for the trans-inhibition assay were set up similarly, and each dilution of the protein was incubated with the specified SRR peptide (23–580 µM) at 4 °C for 10 min. Radiolabeled DNA was added, and the reaction incubated for another 45 min at 4 °C and then resolved on an 8% (wt/vol) native polyacrylamide gel. Radiolabeled DNA was quantitated on dried gels by phosphorimaging. Equilibrium dissociation constants (K_D values) were determined by nonlinear least squares fitting of the free protein concentration [P] vs. fraction of DNA bound ([PD]/[D]), to the equation [PD]/[D]_i = 1/[1 + (K_D/[P])] using KaleidaGraph (v. 3.51; Synergy Software). Due to the low concentration of total DNA, the free and total protein concentrations are effectively equal. Reported K_D values represent the average of at least two independent experiments ± SEs of the means.

**NMR Spectral Assignments.** NMR data were recorded at 25 or 28 °C on 500-MHz Varian Unity, cryoprobe-equipped 600-MHz Varian Inova, and cryoprobe-equipped 600-MHz Bruker Avance III spectrometers. Proteins and peptides were in NMR sample buffer (20 mM Mes, 50 mM NaCl, 0.5 mM EDTA, 0.02% NaN3, and 5 mM DTT at pH 6.5) with 5–10% lock D2O. Data were processed and analyzed using NMRpipe (1) and Sparky (2). Signals from backbone and sidechain \(^{1}H, ^{13}C,\) and \(^{15}N\) nuclei were assigned by standard multidimensional heteronuclear correlation experiments.

**NMR-Monitored Titrations.** Peptide–protein interactions were monitored via sensitivity-enhanced \(^{15}N\)-heteronuclear single quantum correlation (HSQC) spectra. Experiments involved titrating up to 487 µL of unlabeled peptide (initially 1.7–2.2 mM) into \(^{15}N\)-labeled ΔN301 (initially 450 µL, 0.25 mM), or up to 589 µL of unlabeled ΔN301 (initially 1–2 mM) into \(^{13}C/^{15}N\)-labeled peptide (initially 500 µL, 0.125–0.25 mM). Chemical shift perturbations (CSPs) were calculated from the combined amide \(^{1}H\)N and \(^{15}N\) shift changes as

\[
\Delta\delta = \sqrt{(0.2 \times \Delta \delta^N)^2 + (\Delta \delta^HN)^2}. \quad \text{Equilibrium dissociation constants (K_D values) were determined by fitting, with GraphPad Prism, \(\Delta\delta_i\) to the following equation for a 1:1 binding isotherm:}
\]

\[
\Delta\delta_i = \frac{\left( |A|_T_1 + |B|_T_1 + K_D \right) - \sqrt{\left( |A|_T_1 + |B|_T_1 + K_D \right)^2 - 4|A|_T_1 |B|_T_1}}{2|A|_T_1}
\]

where \([A]_T_1\) and \([B]_T_1\) are the total, dilution-adjusted concentrations of labeled and unlabeled species, respectively, at each titration point i.

**Backbone Amide \(^{15}N\) Relaxation.** Amide heteronuclear nuclear Overhauser effect (NOE) relaxation data were recorded on a 600-MHz spectrometer at 28 °C (3). Proteins or peptides were 100–300 µM in 20 mM phosphate, 50 mM NaCl, 0.5 mM EDTA, 0.02% NaN3, 5 mM DTT, and 10% D2O at pH 6.5. Heteronuclear \(^{1}H\)\{\(^{15}N\}\}-NOE values were calculated as the ratio of peak intensities in spectra recorded with a 2-s recycle delay and 3 s of \(^{1}H\) saturation vs. with a 5-s recycle delay.

**Paramagnetic Relaxation Enhancement Experiments.** The Quik-Change protocol (Stratagene) was used to generate the gene encoding cysteine-free ΔN279 with the mutations C350A and C416A. A single cysteine was then introduced at the nonnative N terminus (H278C) remaining after thrombin cleavage of the His-tag. The resulting protein was expressed, purified as described above and thoroughly exchanged into DTT-free NMR sample buffer using an Amicon ultrafiltration device. Free sulfhydryl groups were reacted overnight at room temperature with a 10:1 molar ratio of the nitroxide spin label S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)ethyl methanesulfonothioate (MTSL; Toronto Research Chemicals). The chemical modification was verified by MALDI-ToF MS. The spin-labeled ΔN279 was concentrated to 50 µM, and unreacted MTSL was removed by multiple buffer exchanges. \(^{15}N\)-HSQC spectra were recorded for the protein in the paramagnetic and then diamagnetic states, the latter being formed by reduction with 10 mM DTT for 24 h. The reported paramagnetic relaxation enhancement (PRE) values are the amide peak intensity ratios \((I_{param}/I_{diam})\) in the two states.

Fig. S1. SRR sequence conservation. Sequence alignment (and conserved sequence logo; Upper) of residues 244–300 in several vertebrate Ets-1 and Ets-2 orthologs. Serines and threonines are highlighted in green, aspartates and glutamates in red, lysines and arginines in blue, and histidines, phenylalanines, tyrosines, and tryptophans in orange. The identified CaMKII phosphoacceptor serines in Ets-1 are denoted with an *. Aromatic and negatively charged residues are adjacent to Ser251, Ser282, and Ser285, which contribute to Ets-1 autoinhibition (1, 2). This forms repeats of Ser-ϕ-Asp/Glu, where ϕ is a phenylalanine or tyrosine.

CSP and PRE studies define the phosphorylation-dependent intramolecular interaction surface for the WT and 4ϕA-mutant SRR within ΔN279. (A) CSPs for main-chain amides (as well as side-chain amides and indoles; Inset) on phosphorylation of ΔN279 (red lines) and ΔN279-4ϕA (blue histogram). In addition to the large CSPs within the SRR due to these modifications, residues encompassing helix H3 and strand S4 of the ETS domain, as well as the Hi-2H1 and S4/H4 loops linking the IM and ETS domain, are also perturbed due to altered intramolecular interactions with the cis-SRR. The ΔN279-4ϕA mutant showed similar patterns, but generally smaller CSPs, than the WT ΔN279, thus supporting the conclusion that both aromatic residues and phosphoserines contribute to the interaction of the SRR and ETS domain. See also Fig. 1. Amide $^{15}$N-HSQC intensity ratios (Ipara/Idia) for (B) ΔN2790P and (C) ΔN2792P with a paramagnetic MTSL nitroxide spin label disulfide-linked to the N-terminal Cys278 vs. with a reduced diamagnetic (or cleaved) hydroxylamine label. Lower intensity ratios reflect increased PREs due to closer average proximity of the spin label and the amide proton. As expected, most residues in the disordered SRR showed strong PRE effects due to the adjacent spin label. More interestingly, intramolecular PREs were also observed for amides clustered in the loop linking the N-terminal inhibitory sequence to the ETS domain (Hi-2 to H1), the DNA binding helix (H3) and the following β-strand S4, and helix H4 of the IM. Thus, the terminal spin label must at least transiently localize to this region of ΔN279. On phosphorylation, the PRE effects remained localized to residues along the ETS domain DNA-binding interface and slightly less so to the N-terminal inhibitory sequence. Similar effects were seen with a paramagnetic Cu$^{2+}$ bound to an ATCUN motif (Gly-Ser-His) at the N-terminus of ΔN301 (1R36) and help define the intramolecular interaction surface for the WT SRR on the ETS domain. This surface is highlighted by mapping the intensity ratios for residues 301–440 onto the structure of ΔN301 (PDB 1R36) using a color gradient from red to blue for values of 0 (maximal PRE) to 1 (negligible PRE). Gray indicates prolines or residues with missing data due to spectral overlap.

Phosphorylation partially dampens fast backbone motions of the dynamic SRR in ΔN279-4φA. Heteronuclear $^1\text{H}$$^{15}\text{N}$-NOE values of ΔN279-4φA in its unmodified (Top; orange) and phosphorylated (Middle; purple) states collected on a 600-MHz spectrometer at 28 °C. (Bottom) Changes (ΔNOE) on phosphorylation. Heteronuclear NOE values above ~0.6 are characteristic of ordered rigid regions, whereas decreasing values indicate increasing flexibility on the subnsec timescale. With heteronuclear NOE values ~0, the SRR is dynamic but not completely unrestrained, in the context of ΔN279-4φA$^{0P}$. On phosphorylation, the NOE values of the aromatic-free SRR increased to ~0.3, indicating partially dampened motions due to increased intramolecular interactions. This is consistent with the higher affinity of SRR-4φA$^{2P}$ vs. SRR-4φA$^{0P}$ for ΔN301 (Table 3). However, in both cases, the SRR had lower NOE values than the well-ordered ETS domain, confirming the fuzzy dynamic nature of these transient interactions. Similar behavior has been reported for the SRR in WT Ets-1 constructs (1, 2). Missing data correspond to prolines or residues with overlapped signals.

Fig. S4. Trans-inhibition of ΔN301 DNA binding by the SRR peptide requires both phosphorylation and aromatic residues. The effects of the trans-SRR peptides on DNA binding by ΔN301 were measured by EMSA. Shown are the resulting binding isotherms for ΔN301 in the absence ($K_D = 0.12$ nM; blue) and presence of 0.58 mM SRR (SRR$^{2P}$) ($K_D = 0.73$ nM; red). The sixfold increase in fit $K_D$ values indicates trans-inhibition of DNA binding ($t$ test $P = 0.002$). Consistent with their weaker affinity for ΔN301, the SRR, SRR-4φA, and SRR-4φA$^{2P}$ peptides did not significantly affect ΔN301 DNA binding under these experimental conditions (less than twofold difference in fit $K_D$ values; corresponding $P > 0.1$). The data points and associated error bars were obtained from three replicas and fit to a simple binding isotherm with KaleidaGraph.
**Fig. S5.** Interactions of the trans-SRR peptides with ΔN301 measured by NMR spectroscopy. (A) Superimposed $^{15}$N-HSQC spectra of $^{15}$N-labeled ΔN301 with the unlabeled synthetic SRR$^{2P}$ added in protein:peptide molar ratios of 0 (red), 0.25 (orange), 0.50 (yellow), 0.75 (green), 1.0 (cyan), 1.5 (blue), 2.0 (purple), and 4.0 (magenta). Similar spectra were recorded for SRR$^{0P}$, SRR-4φA$^{0P}$, and SRR-4φA$^{2P}$. Selected amide and indole groups showing CSPs due to peptide binding (including those used in the titration curves of Fig. 2B) are labeled. Also shown are the amide and tryptophan indole (Inset) chemical shift perturbations observed in the $^{15}$N-HSQC spectra of $^{15}$N-labeled ΔN301 (~250 μM) on addition of SRR$^{0P}$ (B; 1.0 mM peptide yielding 37% saturation based on the $K_D$ values of Table 3; blue histogram), SRR$^{2P}$ (B; 640 μM peptide, 84% saturation; red line), SRR-4φA$^{0P}$ (C; 1.1 mM peptide, <37% saturation; orange histogram), or SRR-4φA$^{2P}$ (C; 1.1 mM peptide, 78% saturation; purple line). Regions shaded in gray designate α-helices (rectangles) or β-strands (arrows) in ΔN301 [Protein Data Bank (PDB) ID code: 1R36]. These data were used to generate Fig. 4 and demonstrate that the trans-SRR peptides bind to an interface encompassing helices HI-2/H1 and H3, as well as H4 of the ΔN301 IM and ETS domain, respectively. The same general interface for cis interactions was identified through CSP (Fig. 1 and Fig. S2A) and PRE (Fig. S2 B and C) measurements of ΔN279.
Fig. S6. Identification of interfacial residues in the trans-SRR peptides for ΔN301 binding. Assigned $^{15}$N-HSQC spectra of the $^{13}$C/$^{15}$N-labeled SRR peptide in its unmodified (SRR$^{0P}$. A) and phosphorylated (SRR$^{2P}$. B) forms at 28 °C. Asterisks indicate minor signals from amides with altered chemical shifts due to nearby cis. Legend continued on following page.
X-Pro bonds. cis and trans X-Pro conformation assignments were based on the distinctive $^{13}\text{C}^\gamma$ vs. $^{13}\text{C}^\delta$ chemical shifts of the proline residues (1). Also shown are superimposed $^{15}\text{N}$-HSQC spectra of amides (C and D) and partial $^{13}\text{C}$-HSQC spectra of aromatic residues (E and F) on addition of unlabeled ΔN301 to the labeled peptides. These spectra were used to generate Fig. 3 A and B.


Fig. S7. The trans-SRR peptides bind similarly to the ETS domain of ΔN301 as do the SRR residues in the intramolecular context of ΔN279. (A–C) Selected regions of the superposed $^{15}\text{N}$-HSQC spectra of $^{15}\text{N}$-labeled ΔN301 (cyan), $^{15}\text{N}$-labeled ΔN301 with unlabeled SRR$^{2\text{P}}$ (red; 84% saturation), and $^{15}\text{N}$-labeled ΔN279$^{2\text{P}}$ (brown). ETS domain residues in the peptide-bound complex have amide $^{1}\text{H}$-$^{15}\text{N}$ chemical shifts that are colinear between those of ΔN301 and ΔN279, indicating similar perturbations due to the trans- and cis-SRR, respectively. The intermediate chemical shift changes for the complex are attributable to incomplete saturation of ΔN301 with the SRR$^{2\text{P}}$ peptide. Similar results are found for ΔN301 and unmodified SRR peptide relative to ΔN279.
Fig. S8. The SRR is predominantly disordered, even when phosphorylated and bound as trans-peptides to ΔN301 or in the intermolecular context of ΔN279. (A) Shown are normalized propensities of the SRR\textsuperscript{0P} (green), SRR\textsuperscript{2P} (black), and ΔN301-bound SRR\textsuperscript{2P} (∼85% saturation; red) peptides to adopt extended, polyproline helix 2 (PPII), and random coil conformations as calculated from backbone $^{13}$C and $^{15}$N chemical shifts (trans X-Pro only) with δD (1). Similar propensities were predicted for peptides with cis X-Pro conformations. (B) The SRR residues in the cis context of ΔN279 are also disordered without or with phosphorylation. The slight differences in propensities relative to the peptides arise from the lack of $^1$Hα and $^{13}$CO chemical shift assignment for ΔN279 (2). The phosphoacceptor serines (•) and SRR aromatics (ϕ) are identified within the gray-shaded area.

Table S1. Phosphorylation and ΔN301-binding does not markedly change the X-Pro cis/trans conformational equilibria of the SRR peptides

<table>
<thead>
<tr>
<th>Residue</th>
<th>SRR$^{2P}$</th>
<th>SRR$^{0P}$</th>
<th>ΔN301-bound SRR$^{2P}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>V280</td>
<td>20</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>S282</td>
<td>12</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>S285</td>
<td>15</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>A293</td>
<td>18</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>A294</td>
<td>*</td>
<td>15</td>
<td>*</td>
</tr>
<tr>
<td>K299</td>
<td>32</td>
<td>36</td>
<td>33</td>
</tr>
</tbody>
</table>

The listed percentage values = cis/(cis+trans) are derived from the relative intensities of the major and minor amide peaks for each residue in $^{15}$N-HSQC spectra of the SRR peptides and assumed to result from a cis X-Pro bonds of P281, P292, P296, and/or P300. Extracted volumes gave similar results. Residues E289, D290, and L295 also showed multiple signals, but their population could not be quantified reliably due to spectral overlap. cis/trans conformational equilibria is also present in the ΔN279 fragment. Residues R279, V280, P281, S282, Y283, and D284 showed detectable isomerization in the phosphorylated state, whereas residues R279, V280, P281, S282, E289, D290, P292, A293, A294, and L295 presented two different conformations in the unphosphorylated state. However, their relative populations could not be quantified reliably due to spectral overlap of the poorly dispersed $^{15}$N-HSQC signals of the SRR.

*Spectral overlap prevented measurement of cis X-Pro bond populations.*