Multi-phosphorylation of the Intrinsically Disordered Unique Domain of c-Src Studied by In-Cell and Real-Time NMR Spectroscopy

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Dedicated to the memory of Prof. Ivano Bertini in recognition of his important contributions to creating a strong and dynamic European NMR community.

Intrinsically disordered regions (IDRs) are preferred sites for post-translational modifications essential for regulating protein function. The enhanced local mobility of IDRs facilitates their observation by NMR spectroscopy in vivo. Phosphorylation events can occur at multiple sites and respond dynamically to changes in kinase–phosphatase networks. Here we used real-time NMR spectroscopy to study the effect of kinases and phosphatases present in Xenopus oocytes and egg extracts on the phosphorylation state of the “unique domain” of c-Src. We followed the phosphorylation of S17 in oocytes, and of S17, S69, and S75 in egg extracts by NMR spectroscopy, MS, and western blotting. Addition of specific kinase inhibitors showed that S75 and S69 are phosphorylated by CDKs (cyclin-dependent kinases) differently from Cdk1. Moreover, although PKA (cAMP-dependent protein kinase) can phosphorylate S17 in vitro, this was not the major S17 kinase in egg extracts. Changes in PKA activity affected the phosphorylation levels of CDK-dependent sites, thus suggesting indirect effects of kinase–phosphatase networks. This study provides a proof-of-concept of the use of real-time in vivo NMR spectroscopy to characterize kinase/phosphatase effects on intrinsically disordered regulatory domains.

Introduction

Post-translational modification (PTM) is crucial for cells to be able to quickly regulate protein function. Reversible phosphorylation, in particular, is a major regulatory event in signaling pathways. Phosphorylation of serine and threonine residues is the most abundant, and the phosphorylation state at particular sites is the result of a delicate balance between kinases and phosphatases, many of which are also regulated by phosphorylation. The presence of multiple phosphorylation sites in the same protein is quite common. In these cases, the phosphorylation of one site may prevent or enhance the phosphorylation of others, and the order of phosphorylation may determine the final pattern. Thus, the phosphorylation of proteins at multiple sites is best studied in real time and in intact cellular systems. Moreover, the use of proteins produced in bacteria without PTM provides a way to study how the endogenous kinase–phosphatase machinery establishes relevant phosphorylation patterns. Serine and threonine phosphorylation causes very large shifts in nuclear magnetic resonance chemical shifts of the corresponding amide protons because of intra-residue hydrogen bonding between amide protons and phosphate moieties. The non-invasive character of NMR spectroscopy and the selectivity provided by isotopic enrichment allow the recording of spectra of isotope-enriched proteins in living cells. Although steric crowding is expected to cause only minor increases in the correlation time of proteins in the cytoplasm, nonspecific interactions with other cellular components often result in restricted rotation and broad lines for globular proteins. In contrast, local motion in intrinsically disordered proteins or protein fragments usually results in narrow in-cell NMR line widths.

Intrinsically disordered regions (IDRs) are highly abundant in the proteins of eukaryotic organisms, but not in those of prokaryotes, thus suggesting that their existence is linked to the increased regulatory demands of multicellular organisms. Intrinsically disordered proteins (IDPs) combine functional plasticity with a reduced tendency to form amyloids. Indeed, IDRs are often associated with cell regulation and signaling, and 80% of the proteins involved in human cancers contain long IDRs. PTMs sites are often located in exposed, flexible segments and IDRs often display phosphorylation sites.
c-Src is the leading member of a family of non-receptor tyrosine kinases implicated in many cellular functions.\textsuperscript{15–19} The c-Src protein is composed of an N-terminal myristoylated membrane-anchoring region (SH4 domain), followed by an IDR called the “unique domain”, SH3 and SH2 domains, and finally the kinase (SH1) domain.

The unique domain contains several sites susceptible to phosphorylation and dephosphorylation in response to diverse cellular processes.\textsuperscript{20–22} Although phosphorylation of S17 by PKA (cAMP-dependent protein kinase) is a well-characterized process, its biological significance remains obscure.\textsuperscript{23} Other phosphorylation sites within the unique domain that have been identified and are believed to modulate the activity of the enzyme are T34, T46, and S72 in chicken c-Src, which correspond to T37 and S75 in human c-Src (T46 has no equivalent in humans). Phosphorylation of these residues by cyclin-dependent kinase 1 (Cdk1/cdc2) was first observed during mitosis.\textsuperscript{24} S75 is not present in other Src family kinase (SFK) members, although it is conserved in Src of all species. Mitosis-independent phosphorylation of S75 by Cdk5 was observed in neurons and in certain tumor cell lines.\textsuperscript{25} Phosphorylation of Src at S75 by Cdk5 is a crucial mechanism for the regulation of intracellular Src activity, as it is directly implicated in modulating the ubiquitin-dependent degradation of active Src.\textsuperscript{26}

A major regulatory role of the unique domain of c-Src has been recently demonstrated.\textsuperscript{27} The discovery followed observations of a number of intra- and inter-molecular interactions involving the unique domain and lipids or proteins. The biological relevance of these interactions was demonstrated in Xenopus laevis oocytes. In this model system, we were able to show that mutations in residues of the unique domain abolished lipid binding and caused a lethal phenotype in the mature oocyte. Moreover, in vitro studies showed that the phosphorylation of S17, T37, and S75 modulated lipid binding by the unique domain.\textsuperscript{28} These phosphorylation sites are highly conserved in Src of phylogenetically distant species (in particular, human and X. laevis).

Here we present an NMR study of the phosphorylation of the c-Src unique domain, either by injection into Xenopus oocytes, or in the presence of extracts from unfertilized Xenopus eggs, which contain a rich repertoire of kinases and phosphatases. This study constitutes a proof-of-concept of the potential of time-resolved NMR in cells or cell extracts (either alone or in combination with MS and biochemical methods) to study multiple phosphorylation–dephosphorylation events of intrinsically disordered regulatory domains of signaling proteins. The methods presented here allow direct observation of the integration of multiple-phosphorylation regulatory processes into the complex kinase–phosphatase networks active under particular physiological or pathological conditions.

Results and Discussion

The unique domain of c-Src gives well-resolved NMR spectra in Xenopus oocytes: Phosphorylation by endogenous kinases

A \textsuperscript{15}N-labeled construct (“USrc”, the first 85 residues of human c-Src followed by a Strep-tag sequence) was expressed in Escherichia coli and purified as previously described.\textsuperscript{28} This con-

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Figure 1. $^1$H,$^15$N correlation NMR spectra of USrc A) injected into intact Xenopus oocytes, B) in cytoplasmic extract from injected oocytes, and C) dissolved in buffer. Circles mark the position of pS17. The curved line indicates the shift to the left from unmodified (ovals) to phosphorylated S17. D) Amino acid sequence of USrc. Strep-tag residues are in italics.

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struct contains the SH4 and unique domains. The glycine residue following the initial methionine was mutated to alanine to prevent myristoylation after injection into the oocytes. Figure 1 shows \(^{1}H,^{15}N\) correlations of the NMR spectra of USrc, either injected into intact Xenopus oocytes (Figure 1A), in cytoplasmic extract obtained from injected oocytes (Figure 1B), or dissolved in buffer (Figure 1C). Well-resolved NMR spectra were obtained under the three conditions. Most of the resonances of USrc appeared at very similar frequencies, thus making their assignment within the cellular environment straightforward. Comparison of HSQC spectra in oocytes or extracts with those from \(^{15}N\)USrc isolated from cells by Strep-tag affinity purification after the NMR experiments ruled out degradation within intact oocytes, in extracts of Xenopus oocytes, or in unfertilized eggs (results not shown).

In vitro studies have shown that the unique domain contains a secondary lipid binding region, in addition to the SH4 lipid-anchoring moiety.\(^{27}\) The observation of well-resolved spectra in the intact oocyte suggests that interactions involving this secondary site are weak, at least in the absence of myristoylation.

A major difference was the appearance of a new peak, which, on the basis of its chemical shift, corresponded to a phosphorylated serine or threonine.\(^{22}\) The appearance of this peak matched the disappearance of the signal from S17, thus indicating that this serine residue is spontaneously phosphorylated in Xenopus oocytes. Similar results were observed when USrc was added to extracts containing the soluble fraction of lysed oocytes, thus indicating that phosphorylation involved a kinase that is soluble, or at least is associated with the soluble lipid fraction (Figure 2A). S17 can be phosphorylated in vitro by purified PKA,\(^{28}\) and this kinase is known to be active in Xenopus oocytes.\(^{29}\)

Chemical shift changes between \(^{1}H,^{15}N\) HSQC spectra in buffer and in oocyte extracts are shown in Figure 2B. Although most residues displaying small changes were assigned by reference to the spectra obtained in buffer, resonances from residues Q13, R14, R15 (or R16), S17, L18, E19, and A21 were significantly shifted or had disappeared in oocyte extracts and could not be assigned. These residues cluster around S17 and included a number of charged residues that are probably affected by the presence of the additional charge of the phosphate group at S17. Residue E22, another charged residue close to S17, also showed chemical shift changes above the standard deviation (0.13 ppm) of the variations observed between buffer and oocyte extracts. Likewise, the charge-sensitive residue H47 and its neighbor G46 (although located far from S17) were also significantly perturbed in the oocyte extracts. Interestingly, hydrophobic residues A27 and F32 in the intervening region were also affected. USrc phosphorylated at S17 showed only very small perturbations around H47 in buffer,\(^{28}\) thereby suggesting that the perturbations observed in the oocyte extract are mediated by the interaction with some components of the extract. Residues R48, N68, and R78 could not be distinguished in the cell extract due to spectral overlap.

Figure 2. A) Overlay of expansions of \(^{1}H,^{15}N\) HSQC spectra of USrc in buffer (blue) and in Xenopus oocyte extract (orange). Circle marks the position of pS17. B) Chemical shift changes between the two conditions. Red asterisks indicate residues with large perturbation (preventing their unambiguous assignment in the phosphorylated sample). The phosphorylation site is indicated.

**Time-resolved multiple phosphorylations of USrc in Xenopus egg extracts**

Extracts obtained from unfertilized X. laevis eggs may be referred to as cytostatic factor (CSF) extracts. Xenopus eggs are arrested in metaphase II of meiosis, and present a larger number of active kinases than do oocytes, including members of the cyclin dependent kinase (CDK) family.\(^{30}\) We and others have shown that S75 in the unique domain of c-Src can be phosphorylated by active Cdk5, both in vitro and in vivo.\(^{26, 28}\) Figure 3A shows a comparison between \(^{1}H,^{15}N\) HSQC correlation spectra of \(^{15}N\)-labeled USrc in buffer and in Xenopus egg extract.

After prolonged incubation of \(^{15}N\)-labeled USrc in the X. laevis egg extracts, three signals with chemical shifts typical of phosphorylated Ser/Thr were observed at the following \(^{1}H,^{15}N\) chemical shift positions (in ppm): 8.596, 120.48 (peak 1), 8.840, 121.76 (peak 2), and 8.96, 117.72 (peak 3; Figure 3A). However, the three new peaks appeared at different times and at different rates. The NMR spectra correspond to the major species present after phosphorylation and might not include the effects of minor phosphorylated forms. A number of peaks from nonphosphorylated residues changed the chemical shifts. The chemical-shift perturbations of USrc residues in the major
species present after reaching the steady state (3 h after the reaction started) in the egg extract are shown in Figure 3 B.

In order to increase the time resolution in the observation of the multiple phosphorylation events of USrc, we optimized the experimental conditions by using a combination of SOFAST-HMQC (to minimize the recycle time between successive NMR scans) and non-uniform sampling (to achieve good resolution in the indirect dimension in minimal time). [31–33] Under our optimized conditions (see the Experimental Section), we recorded well-resolved 2D spectra with acceptable signal-to-noise ratio every 4–5 min from 50 μM USrc samples in X. laevis egg extracts.

The peak 1 resonance appeared in the spectrum 10–15 min after addition of USrc to the extract. Peaks 2 and 3 were first observed in the spectra 15–20 min after the reaction started. Figure 4 A shows the variation of peak intensities over time, for the unmodified S75 signal and for peak 1. Each intensity point was calculated as a running average of two experiments (5 min each). After adding USrc to the extract, phosphorylation caused a decrease in the intensity of the S75 NH signal (this matched the increase in peak 1) until a plateau was reached at 2 h.

The time evolution of the concentration of phosphorylated Ser/Thr residues of USrc in Xenopus egg extracts contributing to peak 2 and peak 3 intensities are shown in Figure 4 B and C, respectively. The two peaks increased with different rates and more slowly than peak 1. In untreated extracts, the relative intensities of peaks 1, 2, and 3 were 1:0.3:0.5, after around 2 h of reaction.

USrc phosphorylation sites and active kinases in Xenopus egg extracts

In order to assign residues that are phosphorylated in the Xenopus egg extract, we combined NMR, MS, and biochemical methods. The appearance of peak 1 paralleled the disappearance of the signal from unphosphorylated S75, thus suggesting that this peak corresponds to pS75. We previously observed phosphorylation of USrc S75 by Cdk5 activated with p25 in vitro. [28] The phosphorylation of S75 was confirmed in USrc recovered after the NMR experiments with egg extracts by MS by detection of pS75-specific tryptic and chymotryptic peptide MS/MS fragment ions, and by western blotting with a specific anti-phosphoS75-Src antibody (Figure 5).

Treatment of the egg extracts with roscovitine (an inhibitor of several CDKs, including Cdk5) prevented the appearance of peaks 1 and 2, thus confirming that the corresponding phos-
phorylation events were catalyzed by CDKs (Figure 6 A). Cdk1 is known to be active in *Xenopus* egg extracts. [30] However, treatment of the extract with Ro3306 (a selective inhibitor of Cdk1) had no effect on the phosphorylation of USrc, as determined by NMR (Figure 6 B). Western blot analysis confirmed that phosphorylation of USrc at S75 was partially inhibited in *Xenopus* egg extracts upon addition of roscovitine but not of Ro3306 (Figure 6 G).

In vitro incubation of USrc with Cdk5/p25 has been shown to result in phosphorylation of both S75 and T37. [28] However, phosphorylated T37 could not be detected by MS after incubation of USrc with *Xenopus* egg extracts. In contrast, MS unequivocally detected phosphorylated S69, although at lower abundance than pS75 (Figure 5 and Figure S1). Phosphorylation of residue S69 of c-Src had previously been detected by MS in cell extracts from cancer lines HCT116 and MDA-MB-435,[34] although its function is presently unknown. S69 and S75 are present in the same tryptic peptide. Nevertheless, MS detected only mono-phosphorylated forms. The position of the phosphorylation site in these isobaric forms was deduced from exclusive peptide fragments. Given that the parent ion was mono-phosphorylated, fragments singling-out a potential site (phosphorylated or not) could be defined as exclusive fragments (Table S1). A very rough estimate of the phosphorylation levels from the relative fragment intensities suggested a pS69/pS75 ratio of around 0.25:1.

The relative intensity of NMR peak 2 to peak 1 was 0.32, thus suggesting that peak 2 might correspond to pS69. However, the appearance of this peak (assigned to pS69) did not result in an observable decrease in the signal from the unmodified residue. This apparent contradiction could be accounted for by the observation of a general increase in the intensities caused by the phosphorylation of S75, thus masking the small decrease expected from the (minor) phosphorylation

![Figure 5](image1.png)

**Figure 5.** A) Extracted ion chromatograms of m/z 463.2210 (calcld m/z 463.2208, Δm = 0.43 ppm; top) and 896.8997 (calcld m/z 896.8987, Δm = 1.11 ppm; bottom) corresponding to RRpSLEPAENVH [M+3H]3+ and p-LFGGFNSSDTVTSPQR [M+2H]2+ ions, respectively. B) Western blots showing phosphorylation of S17 and S75 of USrc in CSF extracts. Protein loading was determined by using an antibody against the Strep tag. C) The monophosphorylated peptide p-LFGGFNSSDTVTSPQR is a mixture of LFGGFNSSDTVpSPQR and LFGGFNpSSDTVTSPQR; these can be distinguished by their MS/MS transitions: (896.89 → 567.25, y1) and (896.89 → 972.48, y9-H2O), respectively.

![Figure 6](image2.png)

**Figure 6.** A)–F) Expansions of 1H,15N HSQC spectra of USrc in *Xenopus* egg extracts (CSF) treated with CDK inhibitors A) roscovitine and B) Ro3306, or with PKA inhibitors: C) 6-22 fragment and D) H89. E) and F) expansions of 1H,15N HSQC spectra of USrc at 10 and 180 min after addition of H89 following preincubation in CSF for 30 min. G) Effects of CDK/PKA inhibitors on S75 phosphorylation. USrc was isolated after incubation with CSF extracts and roscovitine (0.5 mM), Ro3306 (25 μM), 6-22 (50 μM), or H-89 (3 μM). Membranes were immunoblotted with anti-PhosphoSrc-Ser75 (top) and anti-StrepTag (bottom).
PKA is not the major kinase phosphorylating S17 of USrc in Xenopus egg extracts.

Although S17 can be phosphorylated by PKA, the addition of the PKA-selective inhibitor PKA-I 6–22 (residues 6–22 of the PKA inhibitory protein PKI) to CSF extracts did not impede the appearance of the pS17 signal (Figure 6C). Thus, an alternative kinase in the extract was able to catalyze S17 phosphorylation of USrc. However, the broader-specificity inhibitor H89 (inhibitor of kinases such as S6K1, MSK1, ROCKII, PKB, and p90RSK, in addition to PKA) prevented phosphorylation of S17, thus indicating that the kinase responsible for the phosphorylation of S17 in Xenopus egg extracts is sensitive to H89. Not surprisingly, Ro3306 had no effect on the phosphorylation of S17. However, addition of roscovitine eliminated peak 3 (pS17) in the NMR signal, thus suggesting an indirect effect of CDK inhibition on the phosphorylation of S17. Interestingly, PKA-I 6–22 and H89 (PKA inhibitors) also had a striking effect on the phosphorylation state of USrc residues targeted by CDKs: in the presence of PKA inhibitors the NMR signals corresponding to unmodified S75 were clearly observed, and peaks 1 and 2 corresponding to phosphorylated species were not present (Figure 6C and D). These results suggest that inhibition of PKA might trigger the activity of a phosphatase that reverses (or strongly reduces) the effect of CDKs, thus keeping S75 unphosphorylated.

In order to test this hypothesis, H89 was added after 30 min of preincubation of USrc with the extract to allow the phosphorylation of USrc by CDKs. NMR measurements showed a decrease in the intensity of the CDK-phosphorylated species following addition of H89 (Figures 6E and F), thus indicating that phosphatase activity compensates for the effect of the CDKs in the presence of the PKA inhibitor H89. The amount of phosphorylated S17 remained constant, thus showing that the active phosphatase did not affect pS17. A strong decrease in the phosphorylation of S75 in Xenopus egg extracts upon addition of PKA-I 6–22 or H89 was also observed with a specific pS75-Src antibody (Figure 6G).

A candidate to mediate the effect of H89 is inhibitor-1 (I-1) of protein phosphatase 1 (PP1). This ubiquitously distributed intrinsically disordered protein is phosphorylated by PKA and become a strong inhibitor of PP1. Inhibition of PKA would allow dephosphorylation of I-1 and release of active PP1, thereby leading to the observed absence of phosphorylation of S75.

Consistently, addition of PKA to the extract resulted in an increase in the level of pS75, in addition to complete phosphorylation of S17. Interestingly, addition of PKA prevented the appearance of peak 2 (Figure S3). This effect might indicate that S75 and S69 are targets of a different set of kinases and phosphatases (that have different dependencies on PKA), or it might suggest that phosphorylation of S69 cannot occur after S75 is phosphorylated. Experiments using single point mutants under various conditions are currently in progress.

Conclusions

Real-time NMR spectroscopy allows the study of complex phosphorylation/dephosphorylation processes in the intrinsically disordered unique domain of c-Src: these are mediated by kinases and phosphatases that are present in Xenopus cells and cell extracts. The time-course of the known phosphorylation of S17 and S75 was studied, and the phosphorylation of S69 (not previously described, although detected) was unequivocally characterized by MS and assigned to one of the detected NMR signals. Addition of CDK or PKA kinase inhibitors or of purified PKA clearly showed cross-talk between kinases and phosphatases in Xenopus egg extracts, thus highlighting the benefit of using in vivo NMR to study phosphatase and kinase activities, and their mutual interplay. This approach is highly suited to characterize post-translational modifications in the intrinsically disordered regions that are present in most of the disease-related proteins in eukaryotic cells.

Experimental Section

Cloning and protein expression and purification: cDNA encoding human c-Src (residues 1–85) with a C-terminal Strep-tag for purification purposes was cloned into a pET-14b vector (Novagen/Merck Millipore). The plasmid was transformed in E. coli Rosetta (DE3) pLysS cells (Novagen), and cells were grown in M9 minimal medium supplemented with [15N]H4Cl (Cambridge Isotope Laboratories, Andover, MA). USrc protein was isolated by using Streptactin Sepharose (IBA, Göttingen, Germany). After elution with of desthiobiotin (2.5 mM), it was further purified by size-exclusion chromatography (Superdex 75 26/60, GE Healthcare) in phosphate buffer (50 mM, pH 7.0) with EDTA (0.2 mM).

Xenopus oocytes: X. laevis ovaries were surgically removed from full-grown females and treated with collagenase and dispase. [60] Stage VI oocytes were selected and maintained in modified Barth’s saline (MBS). Oocytes were microinjected with purified [15N]-labeled USrc protein (50 nL, 1 mM) and left to recover in modified Barth’s medium (3 h, 18 °C) before NMR measurements.

For the preparation of lysate, oocytes were homogenized in ice-cold H1K buffer ([β-glycerophosphate (80 mM, pH 7.5), EGTA (20 mM), MgCl2 (15 mM), DTT (1 mM), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 mM], benzamidine (2.5 mM), aprotinin

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(10 μg·mL⁻¹), and leupeptin (10 μg·mL⁻¹); 10 μL per oocyte). Lyase was centrifuged (10000 g, 10 min, 4 °C), and the cleared supernatant was used for NMR measurement. CSF extracts were prepared from unfertilized X. laevis eggs as previously described, [26, 27] and immediately stored as aliquots (100 or 200 μL) at −80 °C.

**NMR sample preparation:** Cell-extract samples for NMR experiments were prepared by mixing 15N-labeled USrc protein (25 μL, 0.5 mm stock solution; in phosphate buffer (50 mm, pH 7.0), EDTA (0.2 mm) and D₂O (25 μL, 10%)) with CSF or oocyte extract (200 μL). Prior to NMR measurement, CSF extracts were thawed on ice and quickly spun down. In order to minimize time for temperature equilibration in the spectrometer, D₂O and 15N-labeled protein stock solutions were kept at 16–18 °C before being added to the extract. Immediately after adding protein to the extract, the samples (final volume 250 μL) were transferred to Shigemi tubes and inserted in the spectrometer for measurements.

The in-cell NMR samples were prepared as described above and previously. [28] After recovering (see above), oocytes were washed twice with MBS and, 30 min before NMR measurement, transferred to MBS containing D₂O (10%). A stock solution of 15N-labeled USrc protein (50 μL, 1 mm) was microinjected in each oocyte to provide an in-cell concentration of around 50 μM—assuming that the intra-cellular volume of an oocyte is around 1 μL. Two hundred and fifty oocytes were used for individual in-cell NMR samples.

The following products were used for the different in vivo assays: Protein kinase A (P2645–4000U, Sigma–Aldrich), H89 dihydrochloride (#2910, Tocris Bioscience), protein kinase A inhibitor fragment 6–22 amide (P6062, Sigma–Aldrich), Ro3306 (#4181, Tocris Bioscience), and roscovitine (#557360, Calbiochem/Merck Millipore). Prior to addition of 15N-USrc, the kinase inhibitors were incubated for 30 min with CSF extract.

**Western blotting:** USrc was recovered from CSF extracts by Strept- ag affinity purification after NMR measurements. Purified protein (0.5 μg) was separated by SDS-PAGE and transferred to Hybrid ECL nitrocellulose membrane (GE Healthcare). Membranes were blocked with milk (5% in TBS-Tween) and immunoblotted with the following antibodies: anti-PhosphoSrc-Ser75 (1:20 000; IBA), anti-PhosphoSrc-Thr383 (1:1000; #8A8186, AAT Bioquest, Sunnyvale, CA), and anti-Streptag (1:2000; Calbiochem/Merck Millipore). Prior to addition of 15N-USrc, the kinase inhibitors were incubated for 30 min with CSF extract.

**Mass spectrometry:** 15N-labeled USrc (20 μg) recovered from NMR experiments (or unlabeled USrc treated in an identical way) was digested by adding trypsin or chymotrypsin (2% w/v) and incubated at 37 °C overnight. Digestion was stopped by adding formic acid (FA, final concentration 1%). The resulting peptide mixtures were diluted in FA (1%) and loaded into a nano-LC-MS/MS system connected to a TriVersa NanoMate (Advion, Ithaca, NY) fitted to an LTQ-FT Ultra mass spectrometer (Thermo Scientific). Further experimental details are given in the Supporting Information.

A database search was performed with Bioworks (v3.1.1, SP1, Thermo Scientific) and Proteome Discoverer (v1.3, Thermo Scientific) by using the Sequest search engine and a home-made database (SwissProt format) that included the USrc protein, and the common repository of adventitious proteins (http://www.thegpm.org/crap/index.html). Search parameters included no-enzyme specificity, methionine oxidation and phosphorylation in serine and threonine as dynamic modifications, and, depending on the sample, amino acids labeled with 15N as static modification.

Extracted ion chromatograms of MS or MS/MS ions were obtained by using Xcalibur software (v2.0 SR2, Thermo Scientific).

**NMR spectroscopy:** NMR experiments with 15N labeled USrc (0.5 mm in phosphate buffer (50 mm, pH 7.0)) were recorded at 288 K with a 600 MHz Bruker Avance III spectrometer equipped with a TCI cryo-probe. NMR experiments with live oocytes or cellular extracts were performed at 288 K (optimum for live oocytes), and USrc (50 μM).

USrc resonance assignment has been previously reported. [29] Combined NH chemical shift differences were evaluated with Equation (1):

\[
\Delta \delta = \Delta \delta^H + (\Delta \delta^N/5)^{0.5}
\]

where δₜ and δₙ are the changes in chemical shift for H and N, respectively. In vitro kinase reactions were reconstituted with 15N-USrc (50 μM) and recombinant PKA (400 U) in the presence of ATP (0.5 mm) and MgSO₄ (0.1 mm).

NMR experiments with buffer or cells were performed in Shigemi tubes (Shigemi Inc., Allison Park, PA). For experiments with cell extracts Shigemi or 3 mm NMR tubes were used. Probe tuning and shimming was performed in a dummy sample equilibrated at 288 K immediately before insertion of the sample, to minimize dead-time in the real-time measurements. The time from protein addition to the start of NMR data acquisition was 5 min. For quantitative time-resolved experiments, we acquired consecutive 1H, 15N SOFAST-HMQC [31] correlation spectra while the kinase reaction proceeded. The data were recorded with four or eight transients and 800 complex points for 1H and 256 or 512 complex points for the 15N dimension by using traditional or non-uniform sampling schemes. Spectral width in the 15N dimension was minimized in order to improve resolution in the indirect dimension. NMR data were processed with NMRPipe [30] or qMDD [32] software and spectra were analyzed with SPARKY [33].

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