Autophosphorylation activates c-Src kinase through global structural rearrangements

The prototypical kinase c-Src plays an important role in numerous signal transduction pathways, where its activity is tightly regulated by two phosphorylation events. Phosphorylation at a specific tyrosine by C-terminal Src kinase inactivates c-Src, whereas autophosphorylation is essential for the c-Src activation process. However, the structural consequences of the autophosphorylation process still remain elusive. Here we investigate how the structural landscape of c-Src is shaped by nucleotide binding and phosphorylation of Tyr516 using biochemical experiments, hydrogen/deuterium exchange MS, and atomistic molecular simulations. We show that the initial steps of kinase activation involve large rearrangements in domain orientation. The kinase domain is highly dynamic and has strong cross-talk with the regulatory domains, which are displaced by autophosphorylation. Although the regulatory domains become more flexible and detach from the kinase domain because of autophosphorylation, the kinase domain gains rigidity, leading to stabilization of the ATP binding site and a 4-fold increase in enzymatic activity. Our combined results provide a molecular framework of the central steps in c-Src kinase regulation process with possible implications for understanding general kinase activation mechanisms.

The human kinome includes 518 kinases, most of which show very diverse mechanisms of activation even when closely related (1). For some kinases, activation is tightly linked to substrate binding by establishing a SH2–kinase–substrate interface, as in the case of Fes and Abl (2). Other pathways include a dimerization step, as for SHK, LOK, DAPK3, and CHK2 (3). Again, other kinases are activated by a binding partner, as is true for the p38α–TAB1 pair and CaMKII and calmodulin (4, 5). However, a general element during the activation process is phosphorylation of the activation loop, leading to the stabilization of the active conformation.

The tyrosine kinase c-Src is one of nine highly related Src family kinases and the most oncogenic. It is ubiquitously expressed in all tissues (6) and plays an important role in numerous signal transduction pathways ranging from cell proliferation to growth and survival. Deregulation of c-Src activity is involved in cancer emergence and progression (7–9) and formation of metastases (10, 11).

c-Src consists of a unique domain, followed by the regulatory SH3 and SH2 domains (RD) and a kinase domain (KD), which is divided into the N-lobe and the C-lobe (Fig. 1A). When phosphorylated by the C-terminal Src kinase, the resulting pTyr527 is bound by the SH2 domain, which leads to a compact conformation and an inactive state (12–16). In contrast, in the activated state of c-Src, this site is dephosphorylated (17). However, Tyr527 dephosphorylation is not sufficient for full kinase activation (18). The KD also carries another phosphorylation site within the so-called activation loop (A-loop), at position Tyr416, which is located at the interface between the two kinase domain lobes. Upon autophosphorylation of this site, kinase activity is increased (19–23).

The compact, inactive conformation is mediated by the interaction between the SH2 domain and pTyr527 as well as binding of the SH3 domain to the SH2 domain kinase domain linker, which imitates the proline-rich sequence of target molecules (24). Consequently, potential substrate binding sites on the SH2 and SH3 domains are blocked, and within the active

The abbreviations used are: SH, Src homology; RD, regulatory domain; KD, kinase domain; A-loop, activation loop; MD, molecular dynamics; H/DX, hydrogen/deuterium exchange; FLP, full length phosphorylated; FLU, full length unphosphorylated; KDU, kinase domain unphosphorylated; KDP, kinase domain phosphorylated; ANS, 1-anilino-8-naphthalenesulfonate; AMP-PNP, adenosine 5'-[(β,γ-imino)triphosphate].

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center, the αC-helix is tilted, leading to a conformation incompatible with formation of an important salt bridge between Lys295 and Glu310; thus, enzymatic activity is inhibited (25). In addition, the A-loop forms an α-helix that sterically blocks the active center (24). The activation process of c-Src is divided into three parts. Substrate proteins displace both pTyr527 and the linker from the SH2 and SH3 domains (1). Elimination of the SH2–pTyr527 and the SH3–linker interaction (in a process called unlatching) leads to a conformational change in the kinase domain, resulting in activation of the enzyme (called unclamping) (24) (2). In this conformation, the released C-terminal pTyr527 can be dephosphorylated; the αC-helix is tilted toward the active center, forming the Lys295–Glu310 salt bridge, and the A-loop becomes unstructured (6, 17). Alternatively, activation can be initiated by dephosphorylation of pTyr527 by tyrosine phosphatases (26) (3). In a step called switching, Tyr416 is phosphorylated, and the A-loop can form a platform for substrate binding (17, 24, 27). In this active state, the relative motion of the two lobes of the kinase domain enables the kinase to adopt both an open and a closed conformation. Generally, intramolecular interactions maintain an inactive and external interactions, e.g. with proteins binding to the SH2 or SH3 domains promoting an activated state (28). In the open conformation, ATP can be bound and/or ADP can be released. The closed form is necessary for the active center to coordinate the substrates and to adopt a catalytically competent state (25).

Although the first unlatching and unclamping steps of the activation process are well understood (17, 24), the mechanism of the final switching step and the underlying structural changes upon phosphorylation at Tyr416 remain elusive. Structures without phosphorylation (17) and with phosphorylated Tyr416 (29) shed some light on the structural changes involved. However, in vivo c-Src is either phosphorylated at Tyr527 or Tyr416. A nonphosphorylated kinase comparable with the existing crystal structure has not been observed in vivo (17). Also, the pTyr416-phosphorylated kinase domain was crystallized without its regulatory domains, which are thought to affect its conformation (29). In addition, key parts of the kinase, including the A-loop and pTyr416, were not resolved. Molecular dynamics (MD) simulations and NMR studies indicate that the kinase domain becomes more compact upon phosphorylation at Tyr416 (30, 31), but again these experiments were performed without the regulatory SH3 and SH2 domains. Here we analyze the structural and functional properties of c-Src in the presence or absence of Tyr416 phosphorylation using biochemical and biophysical approaches as well as hydrogen/deuterium exchange (H/DX-MS) and MD simulations to elucidate the final switching step of c-Src kinase.

**Results**

**Tyr416 phosphorylation increases the activity of c-Src**

Tyr416 is part of the activation loop of c-Src, which is located in the C-terminal lobe of the kinase domain (Fig. 1A). To determine how phosphorylation at Tyr416 influences the kinase, we purified the protein from insect cells and performed autophosphorylation in vitro. Mass spectrometric analyses reveal that phosphorylation of Tyr416 is quantitative (Fig. S1A), which was also confirmed later by H/DX-MS measurements in which only expected peptides for the respective phosphorylation states were found. We compared the kinase activity of WT, full-length c-Src phosphorylated on Tyr416 (FLP) with the Y416F variant (c-SrcY416F), which cannot be autophosphorylated. This mutant has been used previously to investigate c-Src activity in vivo (32). We found that, upon phosphorylation of Tyr416, the kinase activity of c-Src was strongly increased.}

Figure 1. Tyr416 phosphorylation increases c-Src kinase activity. A, structure of c-Src kinase (PDB code 1Y57). Coloring from the N to the C terminus: SH3 domain in yellow, SH2 domain in green, linker in gray, and kinase domain in blue. Important elements involved in kinase activation are depicted in red: Tyr416 (spheres), Tyr527, A-loop, KER residues, and HRD motif. B, time-dependent substrate phosphorylation by c-Src full-length in its phosphorylated state (FLP) and the non-autophosphorylatable mutant FLU-Y416F. Note that this is the only experiment in which the Y416F mutant of c-Src was used. Inset, K_m (ATP) for the two different constructs.
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Figure 2. Bioanalytical characterization of different c-Src constructs. Shown are c-Src full-length in its unphosphorylated (orange) and phosphorylated state (red) and the kinase domain of c-Src in its unphosphorylated (dark cyan) and phosphorylated state (navy blue). A, aggregation of c-Src constructs at 42°C was analyzed by absorbance at 350 nm. B, CD spectra of the different constructs. C, stability and unfolding cooperativity measured by CD spectroscopy. Thermal transitions were recorded at 207 nm. Inset, the temperature ranges spanning 10% to 90% of the protein unfolding signal. D, analysis of ANS binding to hydrophobic protein patches. Fluorescence spectra after excitation at 380 nm were recorded and buffer-corrected. E, tryptophan fluorescence emission quenching using acrylamide. Fluorescence signals at 328 nm were recorded after excitation at 295 nm. All measurements were performed at least in triplicate. d.u., arbitrary units.

Increased, with FLP showing a 3.7-fold higher $k_{cat}$ relative to c-SrcY416F (Fig. 1B). The affinity of c-Src for ATP decreased from 17.0 μM for c-SrcY416F to 9.8 μM for FLP (Fig. 1B), leading to a 6.4-fold increase in $k_{cat}/K_m$, indicating increased catalytic efficiency. These findings suggest that Tyr$^{316}$ phosphorylation can modulate c-Src dynamics.

The kinase domain is stabilized upon Tyr$^{416}$ phosphorylation

To gain further insight into the activation process, we determined the biophysical properties of full-length c-Src in its unphosphorylated (full length unphosphorylated (FLU)) and its phosphorylated state (FLP) (see “Materials and methods”). To address the role of the RDs in this context, we also studied the isolated kinase domain in the unphosphorylated (kinase domain unphosphorylated (KDU)) and in the Tyr$^{416}$-phosphorylated form (kinase domain phosphorylated (KDP)). Note that all following experiments were performed with constructs based on WT c-Src. Analysis of the aggregation propensity at elevated temperature (42 °C) revealed that the KD is less prone to aggregation than full-length c-Src, independent of its phosphorylation state (Fig. 2A). The FLU construct readily aggregated, and phosphorylation (FLP) enhanced this effect (Fig. 2A). These results indicate that phosphorylation of Tyr$^{416}$ affects the conformation of the RDs, leading to a higher aggregation propensity mediated by the RDs.

c-Src is a protein rich in α-helices, as indicated by the minima at 207 nm and 222 nm in the near-UV CD spectrum (Fig. 2B).

Upon phosphorylation of Tyr$^{416}$ (FLP), c-Src showed a subtle gain in secondary structure (Fig. 2B). We probed whether this difference resulted from a structural transition in the KD. Here also, upon phosphorylation, a higher content of secondary structure was observed (Fig. 2B), suggesting that the observed structural transitions take place in the KD of c-Src. To test whether the conformational changes affect protein stability, we performed thermal transition experiments. We found that all c-Src constructs were similarly stable, independent of their phosphorylation state (Fig. 2C), but the unfolding cooperativity of the KD was slightly increased relative to KDU. This was reflected by a larger temperature range of unfolding (Fig. 2C, inset). Taken together, Tyr$^{416}$ phosphorylation of c-Src does not globally alter the fold of the kinase but increases folding cooperativity in the KD.

To further examine the influence of phosphorylation on the structure of the kinase, we analyzed the surface-exposed hydrophobicity of c-Src by measuring the binding of the fluorophore ANS to hydrophobic patches on the protein surface. The FLU construct showed a strong fluorescence signal, indicating that the kinase exposes a hydrophobic surface area (Fig. 2D). Upon Tyr$^{416}$ phosphorylation, the fluorescence signal decreased slightly. KDU showed much less exposed hydrophobic surface compared with the full-length construct. Interestingly, upon phosphorylation, the ANS fluorescence of the KD (KDP) was decreased (Fig. 2D). This indicates that the RDs contribute significantly to the surface hydrophobicity of c-Src. These data suggest that conformational transitions decrease the accessible hydrophobic area of the KD but that this is compensated in the FL constructs by structural rearrangements of the RDs.

To gain further insight into conformational changes, we analyzed the accessibility of tryptophan residues. In Src kinase, the tryptophan residues are widely distributed across the protein and thus are excellent probes to monitor changes in the global conformation of the kinase. Lower molecular flexibility results in decreased exposure of these hydrophobic residues to quenchers like acrylamide (33). With increasing acrylamide concentrations, we observed a decrease in tryptophan fluorescence, which indicates that a significant fraction of the tryptophans in c-Src are accessible (Fig. 2E). For the FLU and FLP constructs, about 60% of the initial fluorescence could be quenched by acrylamide. The KD exhibited generally less
quenching compared with the full-length kinase, with KDU showing about 30% accessibility. Notably, phosphorylation of the KD (in KDP) prevented tryptophan quenching (Fig. 2E), suggesting that the tryptophan residues are more buried in this variant. Taken together, we conclude that phosphorylation of Tyr416 in c-Src does not significantly affect its secondary structure but seems to strongly influence the dynamics and/or the tertiary structure of c-Src. Although the KD adopts a more rigid conformation upon phosphorylation, the RDs seem to exhibit increased conformational flexibility, causing a higher aggregation propensity.

**Tyr416 phosphorylation globally alters intramolecular interactions**

To understand the molecular basis of these structural and dynamic changes in Src, we applied H/DX-MS (Fig. 3A). This technique provides information regarding local flexibility and dynamics based on amide backbone proton/deuteron exchange with the solvent in a quantitative manner. Rigidity and solvent protection of certain regions within a protein results in slower H/D exchange. Similarly, higher exchange kinetics point toward loss of structural integrity, increased solvent exposure, as well as increased flexibility. We thus conducted head-to-head analyses of phosphorylated and nonphosphorylated species to resolve changes in protein dynamics.

By comparing the FLU and the FLP states, we found that Tyr416 phosphorylation of c-Src led to decreased backbone proton exchange in the KD and parts of the SH2 domain (Fig. 3B and Fig. S2). In contrast, the SH3 and the unique domain were more prone to proton exchange in the FLP state. Thus, in line with our biophysical analysis (Fig. 2), the H/DX-MS results indicate that the KD gains conformational rigidity and increased stability, whereas the regulatory domains seem to open up and become more dynamic upon autophosphorylation of c-Src. Notably, all elements important for kinase activation showed a decrease in solvent accessibility upon autophosphorylation (Fig. 3B): the activation loop, the KER and HRD motifs (34), and the hydrophobic spine.

We then compared the H/D exchange between the FLU and the KDU constructs (Fig. 3C) and found that, upon deletion of the RDs in KDU, the N-lobe of the KD exchanged more protons with the solvent. Because our thermal transition experiments showed no significant loss of stability of this construct (Fig. 3B), this suggests loss of intramolecular interaction in this region and points toward cross-talk between the RDs and the KD, suggesting that the RDs stabilize the catalytic domain. When comparing KDU with KDP by H/DX-MS, we found that proton exchange at the active site was strongly decreased when Tyr416 was phosphorylated, even in the absence of the RDs (Fig. 3D).

To gain insight into the specificity of the allosteric regulation by Tyr416 phosphorylation, we also investigated the well-described Tyr527-phosphorylated state (FLP527) with the FLU state in a head-to-head analysis using H/DX-MS. To this end, we phosphorylated Tyr527 using C-terminal Src kinase and confirmed the phosphorylation by MS/MS (Fig. S3A). Surprisingly, we found only minor alterations in H/D exchange between the FLU and FLP527 states (Fig. S3B). Slightly increased H/D exchange in the FLP527 state was detected in the C terminus, in the middle parts of the KD, and, to some extent, in the SH2 and SH3 domains. Slightly less exchange occurred in regions known to be engaged in intramolecular interdomain interactions in the FLP527 state (15) (Fig. S3B). Notably, the magnitude of all of these changes was much lower relative to those observed for the FLP/FLU comparison (Fig. 3B). These observations suggest that the changes in kinase dynamics observed upon Tyr416 phosphorylation are of specific importance for the structure and function of c-Src.

To obtain a comprehensive picture, we also analyzed H/DX-MS how nucleotide binding influences FL c-Src. We used the nonhydrolyzable ATP analog AMP-PNP to prevent Tyr416 phosphorylation during the experiment. We found that, in the presence of AMP-PNP, the active site within the N-lobe of the Src KD showed a decrease in H/D exchange (Fig. 3E). This finding supports the notion that nucleotide binding is important for triggering a conformational transition toward the active state (35) (Fig. 5A). In stark contrast, the nucleotide increases the H/D exchange in the FLP construct in the SH2 domain and parts of the KD N-lobe (Fig. 3F). This suggests that Tyr416 phosphorylation reshapes the ATP-binding site and may explain the observed increase in ATP affinity (Fig. 1B).

**The dynamics of key residue interactions are changed upon kinase activation**

To better understand the strong effects of Tyr416 phosphorylation on the structure and activity of c-Src, we analyzed the dynamics of the kinase by atomistic MD simulations of the FLU, FLP, KDU, and KDP constructs. Because motifs important for kinase activation were found to show less H/D exchange in the phosphorylated state, we calculated the solvent-accessible surface area of both the HRD motif (His384, Arg385, Asp386), which stabilizes the active state, and three key residues located at the active center (Lys295, Glu310, Arg409 (KER)) along the simulation trajectories in the different states. We found that the HRD motif was less solvent-exposed in the MD simulations when Tyr416 was modeled in a phosphorylated state, suggesting that the KD undergoes a conformational transition to a potentially more active state. The KER motif underwent a minor conformational change between the different phosphorylation states, but this motif nevertheless showed a significant difference between the FL and KD constructs that might result from the interactions between the KDS and the RDs (Fig. 4A). Consistent with previous MD simulations of the isolated KD (30), we found that, in FLP, pTyr416 strongly interacted with a charged cluster of arginines: Arg385 (as part of the HRD motif), Arg409, and Arg419 (Fig. 4, A–C). Moreover, tilting of the αC-helix suggested that the KD is further stabilized in the pTyr416 state, an effect that we observed in simulations of both the full-length and the kinase domain (Fig. 4, C and D). More specifically, we found that the dynamic flexibility of the αC-helix was affected by the formation of an ion pair between Glu305 of the αC-helix and Arg419 of the A-loop in the FLP state that seems to have a stabilizing effect on this region. This interaction is, however, absent in the FLU state, in which Glu305 forms interactions with Arg95 of the RD, and only transiently formed in the FLU/ATP simulations (Fig. 4, E and F).
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A

HDX-MS

FLU vs. FLP

FLP

FLU

KDP vs. KDU

KDU

E

FLU vs. FLU+AMP-PNP

FLU

FLU+AMPPNP

F

FLP vs. FLP+AMP-PNP

FLP

FLP+AMPPNP

SH3-domain

SH2-domain

Kinase Domain

Active Site

Difference (Da)

N-lobe

C-lobe

K295/E310

HRD

A-lope

R409

K295/E310

HRD

A-lope

R409

K295/E310

HRD

A-lope

R409

K295/E310

HRD

A-lope

R409

K295/E310

HRD

A-lope

R409

K295/E310

HRD

A-lope

R409

K295/E310

HRD

A-lope

R409
The RDs have rich dynamics, and we therefore do not expect to sample the full conformational space and interactions with the KD during the microsecond MD simulations. Nonetheless, the strong alterations in the dynamics and the solvent exposure in the RDs suggest that they communicate with the KD.

Our MD simulations reveal that, at the domain interface, residues Arg\textsuperscript{315} and Arg\textsuperscript{316} in the KD N-lobe of the FLU state could bind to Glu\textsuperscript{97} of the SH3 domain (Fig. 4G), which led to the sampling of more compact structures. However, in the absence of the RDs, where these contacts cannot form (KDU simulations), this led to increased solvent exposure of the N-lobe, consistent with the observed changes in our H/DX-MS experiments (Fig. 3C). Upon phosphorylation of Tyr\textsuperscript{416}, Arg\textsuperscript{409} strongly interacted with pTyr\textsuperscript{416} and carboxylates in the A-loop in the FLP and FLP/ATP states, which rendered Arg\textsuperscript{409} unavailable for the contacts with the RDs (Fig. 4H). This effect was observed consistently during two independent simulations (Fig. S4). Hence, together with the H/DX-MS and the biochemical data, these results indicate that there is phosphorylation state–dependent interdomain cross-talk between the KD and the RDs in c-Src.

We have shown previously that ATP binding by the FLU state facilitates transition to the active state in c-Src. This involves opening of the Lys\textsuperscript{295}–Glu\textsuperscript{310} ion pair in the FLU/ATP complex and restoring it in the FLP state and extension of the A-loop in the active FLP state (35) (Fig. 5A). In our simulations, we found that nucleotide binding in the FLP state further led to higher dynamic flexibility of the Glu\textsuperscript{310}–Lys\textsuperscript{295} ion pair, whereas, in the FLU state, this region remained more rigid (Fig. 5, A and B). The simulations indicate that the conformation of the A-loop is also strongly influenced by phosphorylation but largely unaffected by nucleotide binding (Fig. 5, A and B). Although the phosphorylated A-loop behaved similarly in the FL and KD models (Fig. 5B), the complete dissociation of the Glu310-Lys295 ion pair could not be observed in either the phosphorylated or unphosphorylated states of the KD models, whereas, in simulations of the FL/ATP states, different binding modes could be observed. Accordingly, we observed that the ATP-binding site in the FLP state was structurally more open than in the FLU state, suggesting increased k\textsubscript{off} and k\textsubscript{on} rates for the nucleotide in FLP. This conformational shift upon Tyr\textsuperscript{416} phosphorylation might explain the differences observed in H/D exchange of this region and in the activity of c-Src (Figs. 1B and 3, E and F).

**Discussion**

Our study establishes autophosphorylation of c-Src kinase at Tyr\textsuperscript{416} as a crucial regulatory step in the activation mechanism. Autophosphorylation leads to a 4-fold increase in kinase activity and a 2-fold decreased K\textsubscript{m} for ATP. Our integrated structural approach revealed that, in the activated FLP state, the regulatory domains show increased flexibility, whereas the kinase domain gains rigidity. Thus, introduction of a negative charge at position Tyr\textsuperscript{416} has far-reaching structural consequences ranging from local changes to those that affect the entire multidomain kinase.

Upon phosphorylation of Tyr\textsuperscript{416}, all previously identified activation elements show a decrease in H/D exchange, in particular the activation loop (413–419), the active-center KER residues (Lys\textsuperscript{395}, Glu\textsuperscript{410} and Arg\textsuperscript{409}), the HRD motif (His\textsuperscript{384}, Arg\textsuperscript{385}–Asp\textsuperscript{386}), and the hydrophobic spine (His\textsuperscript{384}, Phe\textsuperscript{405}, Met\textsuperscript{314}, and Leu\textsuperscript{325}), which have been shown to stabilize the active state of the kinase. In line with these results, we found, in MD simulations of the isolated catalytic domain, that the HRD motif has decreased solvent accessibility upon Tyr\textsuperscript{416} phosphorylation, which is also supported by earlier studies of the isolated KD (30, 36). In contrast, the solvent accessibility of the KER residues shows little dependence on the phosphorylation state but is reduced in simulations of the full-length protein models. This suggests communication between the RDs and the KD during the activation process and a potential contribution of electrostatic interactions that influence the H/D exchange in these central regions.

CD analyses show that a structural loss in the RDs upon phosphorylation is compensated by a structural gain in the KD in the full-length constructs. The observed loss of folding cooperativity in the KDU construct further indicates that the regulatory domains interact with the catalytic domain and that the resulting control of activity can be specifically modulated by a single phosphorylation event. We found that the RDs stabilize intramolecular interactions within the KD N-lobe, as proposed earlier (29, 37). The deuterium uptake upon phosphorylation correlates with the ANS binding results. Both findings point toward an increase in the dynamics of the regulatory domains, mainly the unique and the SH3 domains. These contain numerous hydrophobic residues whose exposure leads to differences in surface hydrophobicity between the full-length c-Src and its KD alone. In contrast, hydrophobic patches in the KD are shielded upon phosphorylation. This could reflect partial closure of a previously described allosteric ANS binding pocket in the KD of c-Src (38, 39). The increased H/D exchange in the RDs correlates with the observed increase in aggregation propensity of the FLU and the FLP construct compared with KDU and KDP. It thus seems that the RDs, and specifically their

*Figure 3. A, overview of an H/DX experiment. The protein is incubated in an excess of D\textsubscript{2}O buffer, and backbone amide protons are exchanged in a time-dependent manner. After quenching the reaction, the protein is proteolytically digested. Peptides are chromatographically separated and introduced into a QTOF mass spectrometer, where these are additionally separated by ion mobility prior to m/z detection. By measuring time courses (in this study 0 s (reference point), 0.33 s (red), 1 min (orange), 10 min (cyan), 60 min (blue), and 120 min (black)), the mass increase of each peptide is tracked and compared with those derived from a different protein variant in a head-to-head analysis. B–F, H/DX-MS results for comparison of exchange kinetics between different states of c-Src full-length and the c-Src kinase domain; every time point was measured in duplicate. Left panels, the color-coded structure. Red indicates more uptake and blue less uptake of the second-named compared with the first-named state. Regions showing no difference are marked in gray, and undetermined regions are shown in white. Right panels, the sum of differences plots for the different comparisons. Colored lines describe mass differences for each labeling time point, and gray bars represent the sum of differences over all investigated labeling time points for every single peptide (N to C terminus from left to right). The limit of significance (±1 Da), referring to the sum of differences, is indicated as vertical dashed lines (48). Elements important for kinase activation are highlighted. Shown are the active-site KER residues (Lys\textsuperscript{295}, Glu\textsuperscript{310}, and Arg\textsuperscript{409}), the activation loop (Asp\textsuperscript{413}, Arg\textsuperscript{419}), and the HRD motif (His\textsuperscript{384}, Arg\textsuperscript{385}–Asp\textsuperscript{386}).*
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A

B

C

D

E

F

G

H
construct indicates a higher affinity for ATP. Strong changes are indicated by arrows. A and B, interaction probability between phosphorylated and unphosphorylated states. Only changes larger than 10% are shown for clarity. C and D, tilting of the αC-helix in both KD and FL constructs. The reorientation of Arg385 facilitates interaction with Glu305. E–H, difference in interaction probability between phosphorylated and unphosphorylated states. For the FL constructs, only the KD domain is shown. G, contacts between the RDs and the KD are present in FLU for the complete 1-μs trajectory, whereas in the FLP state, rapid dissociation is observed. H, snapshot of the arginine cluster formed upon phosphorylation.

**Figure 5.** A and B, conformational sampling of the central Glu^{110}, Lys^{295} ion pair distance ($d_{E310-K295}$) and the extent of the A-loop ($<d_{A-loop}>$). Both FL and KD constructs with and without ATP are shown.

Our H/D exchange experiments on FLU and FLP reveal pronounced differences in the structural effects on nucleotide binding. AMP-PNP binding led to a decrease in deuterium uptake in the active center region of FLU. These findings are in agreement with the results of our MD simulations, which suggest that binding of ATP lowers the energy barrier and transition from the inactive (FLU) to the active conformation (FLP). These findings are also in line with the observation that the kinase lobes close around a bound ATP molecule (31). In contrast, when we analyzed FLP in the presence of nucleotide, we found increased exchange in the SH2 domain and parts of the KD N-lobe. Generally, changes in the H/D exchange profile of the FLP KD were much less severe compared with those observed for the KD of the FLU. This suggests that phosphorylation strongly alters the behavior of the kinase during nucleotide binding. The MD simulations revealed that the binding site in FLP is more open than in FLU, suggesting that the binding site could be more accessible to ATP. In the FLU state, the active site closes upon ATP binding, which enables the KD to transition toward the phosphorylation-competent state. In contrast, in the FLP state, the ATP binding site is not strongly altered in the presence of a nucleotide. The decreased $K_m$ of this construct indicates a higher affinity for ATP.

Previous studies suggested that Tyr^{416} phosphorylation may affect substrate binding, possibly through an interaction of pTyr^{416} with the neighboring Arg^{409}, similar to a mechanism suggested for Lck (17, 40). Our MD simulations indeed indicate an extended conformation of the phosphorylated A-loop region. In addition, the A-loop sampled more folded states during the FLP simulation, which were stabilized by interactions between the pTyr^{416} and the neighboring Arg^{385}, Arg^{409}, and Arg^{419}. This strong electrostatic hub might explain the pronounced H/D exchange effects independent of the solvent-accessible surface area value, as this has large consequences for the interactions of the involved residues. For example, in the FLP state, Arg^{419} of the A-loop binds to Glu^{305} in the αC-helix; thus, interactions between Arg^{409} and the regulatory domains are broken. These changes driven by Tyr^{416} phosphorylation could therefore explain the observed stabilization of the KD, whereas the regulatory domains become partly destabilized (Fig. 6). Further, we observed a stabilizing effect of the RDs on the kinase N-lobe. We thus conclude that KD::RD domain cross-talk exists in c-Src that is specifically targeted by autophosphorylation. The importance of these results is underlined by the fact that, although the domain assembly in Src completely rearranges in the Tyr^{527}-phosphorylated state, the observed H/DX-MS patterns only differ slightly. It seems that the folding and inherent stability of the domains is not strongly altered by this shift and that, instead, the dynamics are regulated during the second phospo-switch. Combined electrostatic and conformational changes can trigger long-range coupling effects, as observed in the case of c-Src between the regulatory domains and the kinase domain. Similar long-range electrostatically driven conformational coupling effects have been observed recently in other systems (41–43). Thus, our combined data from biophysical experiments and molecular simulations suggest that long-range action-at-a-distance effects might also play a central role in the c-Src activation process.

So far, the role of autophosphorylation in Src activation has not been studied in detail (28). Furthermore, previous studies have mainly focused on the kinase domain of Src (29–31), ignoring the role of the regulatory domains. This study is the first attempt to comprehensively understand the impact of autophosphorylation on the structure and dynamics of the full-length enzyme by using H/DX-MS, MD simulations, and integrated biochemical experiments. Combining the insights...
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Figure 6. Schematic model of c-Src activation upon Tyr416 phosphorylation. The different states during the final c-Src activation process are shown. In the unphosphorylated apo-state (FLU), Lys295 and Glu310 interact strongly, whereas Arg409 in the activation loop binds and stabilizes Tyr416 and the SH3 domain. Upon ATP binding in the FLU/ATP-state, Lys295 is dragged out of its interaction with Glu310 which concomitantly becomes coordinated by Arg409. This leaves Tyr416 free for phosphorylation by another c-Src molecule. In the pTyr416 state (FLP), pTyr416 is complexed by an arginine cluster consisting of Arg409, Arg385, and Arg419, strongly stabilizing the A-loop in an extended conformation. Arg419 fixes Glu305 in the c-helix, which contributes to global stabilization of the kinase domain. Within this state, the Lys295–Glu310 bond is prone to flickering, which, in combination with a stabilized ATP-binding site, contributes to higher ATP affinity. The interaction between Arg409 and pTyr416 prevents binding to the SH3 domain, leading to increased exposure and partial destabilization of the regulatory domains.

Materials and methods

Kinase activity measurements

c-Src full-length, its Y416F mutant, and the c-Src kinase domain were expressed and purified as described previously (45, 46). All constructs eluted as monomeric peaks from the gel filtration column. To achieve full Tyr416 phosphorylation, the kinases were incubated with 10 mM ATP and 5 mM MgCl₂ overnight and subsequently purified using a PD-10 column (GE Healthcare). For activity measurements of c-Src variants, the respective kinase (320 nM) was incubated at 30 °C for 30 min in Src buffer (40 mM Tris-HCl, 150 mM NaCl, 5% glycerin, 5 mM DTT, 10 mM MgCl₂, and 1 mM MnCl₂ (pH 7.5)) supplemented with 40 μM [γ-32P]ATP with an activity of 0.5 Ci. A 10-fold molar excess of acid-denatured enolase (Sigma) was used as a substrate. The reaction was stopped by adding Laemmli buffer and boiling the sample. The samples were separated by SDS-PAGE, and transphosphorylation was detected by applying a phosphorimaging screen onto the gel. The screen was subsequently analyzed using a Typhoon 9200 PhosphorImager and the program Image Quant (GE Healthcare). For the determination of $k_{cat}$ and $K_m$, the initial rates were measured at saturating substrate (Enolase) concentrations and in the presence of increasing amounts of [γ-32P]ATP.

CD spectroscopy and thermal transition

CD measurements were performed using a J-720 spectropolarimeter (Jasco, Grossumstadt, Germany) equipped with a Peltier element. Far-UV CD spectra were measured using 5 μM protein in Src buffer in a 0.1-mm quartz cuvette between 260 and 200 nm. Thermal unfolding of the proteins was monitored at 207 nm, measuring changes of the CD signal between 20 °C and 80 °C at a heating rate of 20 °C per hour. The curves were fitted to a sigmoidal transition.

ANS binding assay

1.6 μM of the respective kinase variant was incubated with 30 μM ANS (Sigma) in Src buffer for 20 min at room temperature.
and subsequently analyzed in a FluoroMax-3 fluorescence spectrometer (Spex) with an excitation wavelength of 380 nm and an emission wavelength of 470 nm. The obtained emission spectra of the proteins were normalized against the Src buffer blank.

**Tryptophan quenching**

Acrylamide (5 mM) was titrated to 500 nM Src kinase in Src buffer at 25 °C and preincubated for 15–20 min at room temperature prior to measurement. No significant influence of preincubation time on the signal could be observed. Tryptophan fluorescence emission quenching upon excitation at 295 nm was recorded using a FluoroMax-3 fluorescence spectrometer (Spex). The slit widths were set to 3 and 5 nm for excitation and emission, respectively.

**Aggregation assay**

Protein aggregation was monitored using a Varian Cary 50 UV-visible spectrophotometer (Agilent) equipped with a temperature-adjustable cuvette holder. Aggregation of 2 μM c-Src was initiated by incubation at 42 °C. The aggregation reaction was monitored at 350 nm over time as an increasing signal caused by turbidity.

**Hydrogen/deuterium exchange–mass spectrometry**

H/DX-MS experiments were performed on a fully automated system equipped with a Leap robot (HTS PAL, Leap Technologies), a Waters Acquity ultra performance liquid chromatography, a H/DX manager, and the Synapt G2-S mass spectrometer as described elsewhere (Waters) (47). The protein samples were diluted in a ratio of 1:10 with deuterium spectrometer as described elsewhere (Waters Technologies), a Waters Acquity ultra performance liquid chromatography, and incubated at 25 °C for 20, 60, 600, 3600, and 7200 s. After the labeling reaction, the protein was denatured, and the exchange was stopped by diluting the labeled protein 1:1 in quenching buffer (100 mM Na₂HPO₄ × 2H₂O, 100 mM NaH₂PO₄ × 2H₂O, 0.5 mM tris(2-carboxyethyl)phosphine, and 4 mM guanidine HCl (pH 2.6) at 1 °C). Digestion was performed online by an immobilized pepsin column (Applied Biosystems, Poroszyme). Peptides were trapped and subsequently separated on a Waters ultra performance liquid chromatography charged surface hybrid C18 column (1.7 μm, 1.0 × 100 mm) with an H₂O plus 0.1% formic acid (v/v) and acetonitrile plus 0.1% formic acid (v/v) gradient. Trapping and chromatographic separation were carried out at 0 °C to minimize back-exchange. Eluting peptides were directly subjected to the TOF mass spectrometer by electrospray ionization. Before fragmentation by MS and mass detection in resolution mode, the peptide ions were additionally separated by drift time within the mobility cell. Data processing was performed using the Waters Protein Lynx Global Server PLGS (version 2.5.3) and DynamX (version 3.0). Each incubation time point was measured in duplicates. The uptake was not back-exchange–corrected because every measurement was performed head to head. For peptides exhibiting significantly differing exchange kinetics in the two compared states, the sum of difference over all time points must exceed a value of ±1.0 Da.

**Molecular dynamics simulations**

Full atomic molecular models of c-Src with and without bound ATP and Tyr₄¹⁹ modeled in a phosphorylated state were constructed based on the X-ray structure of c-Src obtained from the Protein Data Bank (PDB code 1Y57). Each model was solvated in a TIP3P water box with a 100 mM NaCl concentration. The molecular systems comprised approximately 100,000 atoms and were simulated in an NPT ensemble at T = 310 K and p = 101.3 kPa for 1 μs, with an integration time-step of 2 fs using the CHARMM27 force field (49), treating long-range electrostatics with the particle mesh Ewald approach. The conformation of the A-loop was calculated using the average distance backbone oxygen and nitrogen distances between Asp⁴¹³ and Thr⁴¹⁷, Asn⁴¹⁴ and Ala⁴¹⁸, and Glu⁴¹⁵ and Arg⁴¹⁹, as suggested by Meng and Roux (30). All simulations were performed using NAMD version 2.9–2.10 (50) and Visual Molecular Dynamics (51) was used for analysis. The simulation time was 1 μs for all c-Src models.

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