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Conformational rearrangements upon Syk auto-phosphorylation

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Abstract

Syk is a cytoplasmic tyrosine kinase that is activated after recruitment to immune receptors, triggering the phosphorylation of downstream targets. The kinase activity of Syk is controlled by an auto-inhibited conformation consisting of a regulatory region that contains two N-terminal Src homology 2 (SH2) domains inhibiting the catalytic activity of the kinase domain located at the C-terminus. The atomic structure of the related Zap-70 kinase and an electron microscopy (EM) model of Syk have revealed the structural mechanism of this auto-inhibition based on the formation of a compact conformation sustained by interactions between the regulatory and catalytic domains. On the other hand, the structural basis of Syk activation is not fully understood due to the lack of a 3D structure of full length Syk in an active conformation. Here, we have used single particle electron microscopy to analyse the conformational changes taken place in an activated form of Syk induced by auto-phosphorylation. The conformation of phosphorylated Syk is reminiscent of the compact structure of the inhibited protein but significant conformational changes are observed in the regulatory region. These rearrangements could be sufficient to disrupt the inhibitory interactions, contributing to Syk activation. These results suggest that the regulation of the activation of Syk might be modulated by subtle changes in the positioning of the regulatory domains rather than a full opening mechanism as proposed for the Src kinases.

Keywords

single-particle electron microscopy; EM; Syk; ZAP-70; tyrosine kinases

1. Introduction

Syk (Spleen Tyrosine Kinase) is a 72 kDa non-receptor protein tyrosine kinase that associates with activated immune receptors at the cell membrane. Syk is structurally and functionally related to the Zap-70 kinase, and both proteins are considered to be part of the same family of kinases. These kinases are proximal to the cell receptors and their function is the amplification of the activation signals initiated by Src kinases [1,2]. Dysfunction in the Syk family of kinases has been described to cause human diseases [3].

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Initiation of immune signalling pathways is promoted by the recruitment of intracellular Src kinases to immune receptors. Src phosphorylates two immune receptor tyrosine-based activating motifs (ITAMs) located at their cytoplasmic side [4]. Syk and Zap-70 can then engage to phosphorylated ITAMs using two tandem Src homology 2 (SH2) domains located at their N-terminus (Fig. 1A). The atomic structure of the tandem SH2 domains of Syk bound to a double phosphorylated ITAM peptide shows that each SH2 functions as an independent binding module [5]. In contrast, Zap-70 utilises both SH2 domains to recognise just one tyrosine residue [6]. After binding to phosphorylated ITAMs, Syk and Zap-70 are activated by phosphorylation by Src kinases, although Syk can also be activated by auto-phosphorylation [1,7]. Some phosphorylations are important for the regulation of the kinase activity of Syk [8], whereas other phosphorylated tyrosine residues become docking sites for the recognition of targets. For instance, Tyr342 binds to the SH2 domain of Vav1 [9] and Tyr342 and Tyr346 are required for optimal binding to PLC γ [10]. Phosphorylated Syk is an active form of the kinase that can disengage from the immune receptor complex to phosphorylate its targets [2, 11].

The domain structure of Syk comprises a regulatory region at the N-terminus consisting of two SH2 domains, followed by the catalytic kinase domain at the C-terminus (Fig 1A). The two SH2 domains are separated by an inter-SH2 linker forming a helical structure revealed in the atomic structure of the tandem SH2 region of Syk resolved by X-ray crystallography [5]. An inter SH2-kinase linker separates the regulatory region from the catalytic domain. The structure of the kinase domain of Syk has also been solved at atomic resolution in isolation [12]. An atomic structure of full length Syk is presently lacking, whereas a low resolution electron microscopy (EM) model has been solved recently [13]. The EM structure shows that the catalytic and regulatory regions are packed into a compact conformation reminiscent of that found in Src kinases (Fig 1A). Interestingly, this EM model is remarkably consistent with the atomic structure of Zap-70 in its inhibited conformation solved recently by X-ray crystallography [14]. Interactions between the inter-SH2-region with the SH2-kinase linker and the catalytic domain in Zap-70 are found to maintain an inhibited conformation by reducing the flexibility required for catalysis. Interestingly, two tyrosine residues placed in the inter-SH2 domain were found to be critical for preservation of the auto-inhibited conformation. These residues must become phosphorylated by Src kinases to activate Zap-70 [14], and equivalent residues activate Syk after auto-phosphorylation. It is therefore likely that intra-molecular interactions between the regulatory and catalytic regions similar to those described in Zap-70 contribute in Syk to maintain an auto-inhibited conformation [13].

Based on the atomic structure of Zap-70 a model for activation of this family of kinases has been proposed [14]. According to this model, changes in the orientation of the SH2 domains could control the disruption of the inhibitory interactions. These movements could be partially or totally induced by the binding to phosphorylated ITAMs and/or phosphorylation of tyrosine residues. A similar regulatory mechanism is proposed to take part in the related Syk. More recently, it has been shown that different stimuli (phosphorylation by Src, auto-phosphorylation, and substrate binding to the SH2 domains) can equally activate Syk [7] and the accumulation of several of these inputs does not enhance the level of activation. The authors proposed that Syk may function as a switch whereby any of several possible stimuli trigger the acquisition of similar activated conformations.

Our knowledge of the structural basis of Syk activation is limited by the lack of structural data, up to our knowledge, of full length Syk in an active conformation. Here, we have analysed the conformational rearrangements of full length Syk upon auto-phosphorylation using single particle electron microscopy. The comparison between the 3D structures of inactive Syk [13] and the phosphorylated protein reveals significant movements of the regulatory SH2-SH2

region that could disrupt the interactions that inhibit the protein and promote Syk activation, while maintaining a compact conformation.

2. Materials and methods

2.1. Purification of GST-Syk and auto-phosphorylation

Rat GST-Syk was cloned and purified as a GST fusion protein as described before [13,15,16]. Syk phosphorylation was performed by incubating 1 mg/ml of GST-Syk with 1 mM ATP and 2.5 mM MgCl₂ for 60 min at 25 °C. All GST-Syk molecules in the sample were phosphorylated after the incubation, as revealed the mobility shift in an SDS-PAGE.

2.1. Blue native polyacrylamide gel electrophoresis of GST-Syk

Blue native polyacrylamide gel electrophoresis was performed following protocols described recently [17,18]. Electrophoresis was carried out using a 4-15% Tris-HCl gradient gel (Bio-Rad, Cat 161-1122), 15 mM Bis-Tris, 50 mM Tricine, 0.02% Coomassie blue G250 (Serva, Cat 17524) as Cathode Buffer and a 50 mM Bis-Tris solution as Anode Buffer [18]. Samples were loaded into the wells, overlaying the samples in each of them with Cathode Buffer, which contains Coomassie blue G250 (Serva, Cat 17524) for staining of the proteins [17,18].

As a control for the mobility of monomeric GST-pSyk, one aliquot of the sample was denatured extensively in the presence of high concentrations of SDS and heating at 100°C for 15 minutes [18]. Denatured GST-pSyk and native GST-pSyk were run simultaneously in the same blue native gel under native conditions. As markers, several proteins of known molecular weight were employed.

2.3 Electron microscopy and image processing

A few microliters of GST-pSyk were adsorbed to glow discharged carbon coated grids, negatively stained using 2% uranyl acetate and observed in a JEOL 1230 operated at 100 kV at 50000 magnification. Micrographs obtained under low-dose conditions were digitized using a Minolta Dimage Scan Multi PRO scanner at final 10.5 µm step size. An initial model was obtained using the Random Conical Tilt (RCT) method [19] which allows deriving 3D structures from defined views of the specimen. To this end, pairs of micrographs were taken for each area, with and without tilting the specimen holder, selected and processed using the XMIPP platform [20]. Angular refinement was performed using EMAN [21] following similar procedures to those described previously for Syk [13]. The resolution was estimated by Fourier Shell Correlation (*eotest* command in EMAN) to be 22 Å (using the 0.5 cross-correlation coefficient criteria). The density map was visualized using UCSF Chimera [22]. The handedness of the 3D reconstruction of p-Syk was determined by comparison with the hand determined for Syk previously [13], which was the one providing the best fit with the atomic structure of the Zap-70 [14].

The comparison between the 3D structures of GST-Syk and GST-pSyk was performed after the alignment of both reconstructions using commands found in EMAN [21] and XMIPP [20]. To facilitate the observation of the conformational transitions occurring between GST-Syk and GST-pSyk a movie was generated (Supplementary movie S1). The two 3D structures were previously aligned and intermediate volumes created using *morphmrc2mrc.py* also from EMAN [21].

3. Results

3.1 Purification and Electron Microscopy of auto-phosphorylated Syk

We expressed and purified Syk by fusing a glutathione S-transferase (GST) tag at its N-terminus and using baculovirus-infected insect cells as described before [13]. We decided not to remove this tag for the observation of Syk in the electron microscope. The single-particle EM method has a limitation in the size of the molecules that can be typically analysed since molecules with low molecular weight (usually below 100 kDa) are frequently difficult to spot over the noisy background of an electron micrograph. Furthermore, the small size of some molecules can introduce additional difficulties and ambiguities during image alignment and processing of their images obtained in the electron microscope [23]. Consequently, GST-Syk was analysed in the electron microscope to facilitate its observation and latter alignment and processing of its images (Fig. 1). Importantly, the presence of a GST tag has been shown before not to interfere with the biological functions of Syk or its sites of auto-phosphorylation [24, 25], suggesting that the structure and regulation of Syk is not significantly affected by the tag. This finding has been observed also in other non-receptor protein tyrosine kinases [15]. In agreement with these observations, we found that purified GST-Syk was capable of phosphorylating substrates [13].

To prepare phosphorylated GST-Syk (GST-pSyk from now on), the protein was auto-phosphorylated using an *in vitro* kinase reaction. Under the conditions tested we were able to phosphorylate all the protein present in the reaction mixture, as revealed by a complete shift of GST-pSyk in an SDS polyacrylamide electrophoresis (Fig. 1B). This shift was never detected in the absence of ATP or when incubations were performed with ATP in combination with piceatannol, an inhibitor of the kinase activity of Syk (data not shown). Mass spectrometry analysis of the SDS bands for GST-Syk and GST-pSyk detected peptides specifically phosphorylated in the *in vitro* reaction (Fig. 1B). For instance, we detected phosphorylated peptides corresponding to the inter SH2-kinase domain and in the C-terminal tail. Other residues may be also phosphorylated but we did not intend to perform an exhaustive search, since the detected auto-phosphorylation was a sufficient indication of the activation of Syk.

Given that GST tags have been occasionally found to induce the formation of dimers and aggregates of the tagged protein, we tested the oligomeric state of GST-pSyk. Using size-exclusion chromatography, GST-pSyk eluted as a main peak between molecular weight markers of 160 kDa (bovine gamma-globulin) and 44 kDa (chicken ovalbumin) (data not shown), suggesting it behaved mostly as a monomer. To avoid the limitations of the size-exclusion chromatography, we also analyzed the oligomerization of GST-pSyk using blue native polyacrylamide gel electrophoresis. This experiment permits resolving proteins and their complexes under native conditions [17,18]. A molecular weight marker of 90 kDa and denatured GST-pSyk (after extensive heating in the presence of SDS) were used as standards of mobility of a monomeric GST-pSyk. GST-pSyk was found to run under native conditions as a main band with a migration similar to denatured GST-pSyk and therefore corresponding to monomeric GST-pSyk (Fig. 1C). A low abundant band with roughly double size was also detected, suggesting the presence some dimers, but these were clearly a minority (Fig. 1C).

GST-pSyk was adsorbed to carbon-coated grids and negatively stained following similar procedures to those previously used for GST-Syk [13]. Molecules of GST-pSyk were clearly discernible in the micrographs with a distinctive square shape reminiscent of that found for non-phosphorylated GST-Syk (Fig. 1D) [13]. The similarity between the images of phosphorylated and non-phosphorylated GST-Syk was a first indication that Syk phosphorylation did not induce large conformational changes as suggested for the Src kinases [26,27]. The dimensions of these square-shaped molecules were comparable to those of Zap-70 and they were only compatible with a monomeric GST-pSyk. Larger images that could

potentially belong to oligomeric forms of the protein were a minority. These larger images were not selected for further image processing since we wished to avoid heterogeneity in the dataset.

3.2 3D structure of auto-phosphorylated Syk

As a first approach to determine the 3D structure of GST-pSyk we used the Random Conical Tilt (RCT) method [19] on several selected square-shaped views. RCT permits to solve an *ab initio* 3D structure from defined views of a protein on the support film of the EM grid by taking images of the same molecules at zero and ~45-60 degrees by tilting the specimen holder in the microscope. As a disadvantage of the method, the structures show artefact deformations due to several reasons, among them, the technical limitations for tilting the specimen holder of the microscope at high tilt angles. Untilted and tilted pairs of micrographs of GST-pSyk were recorded and processed using XMIPP [20]. A few representative classes of the untilted images were analysed to obtain their 3D reconstruction (Fig. 1E, see a view of one representative RCT structure). The RCT structure of GST-pSyk revealed a square-shaped molecule divided in two distinct structural regions of different size. The overall conformation seemed roughly similar to the structural organization previously observed for non-phosphorylated GST-Syk [13].

To obtain a 3D structure of GST-pSyk that was not affected by the limitations of the RCT method, an independent reconstruction was performed following angular refinement methods similar to those applied before for GST-Syk [13]. The phosphorylated version of GST-Syk was observed in the electron microscope and 5,105 particles were extracted and processed into a 3D structure at 22 Å resolution (Fig. 1F). The structure of GST-pSyk obtained by angular refinement confirmed the overall features of the RCT reconstruction, but, containing a larger coverage of angular sampling, this reconstruction could then be directly comparable with the structure of GST-Syk [13].

This structure revealed that the molecule was divided into a bulky top region flanked by a smaller domain at the bottom (Fig. 1F). These two segments of the protein were contacting at specific points whereas their connections were minimised in other regions of the molecule. When the two faces of GST-pSyk, observed after rotating the molecule 180 degrees, were compared, it became apparent that the degree of interaction between the top and bottom segments of the molecule were more intimate in one face of the protein than the other.

The structure resulting from the selected dataset was found to be compatible only with a monomeric GST-pSyk, supporting the image selection performed. Using an average value for the density of proteins (1.35 g/ml), an estimate of the molecular mass enclosed by this EM reconstruction matched roughly 95 kDa, compatible with one Syk molecule (~70 kDa) plus one GST-Syk (~20 kDa). Overall, the structure of GST-pSyk was found to correspond to a monomer revealing a compact 3D conformation evocative of that found in non-phosphorylated GST-Syk, thus confirming that auto-phosphorylation did not induce dramatic reorganizations in the molecule.

3.3 Domain assignment in GST-pSyk

In order to analyse the conformational changes induced by auto-phosphorylation of Syk, the several structural regions of the 3D reconstruction of GST-pSyk had to be annotated and assigned to specific domains of Syk (Fig. 1A). To this end, we made use of the existing atomic structures of the isolated kinase and regulatory domains of Syk and the structure of full-length Zap-70 to be compared with the EM reconstructions. However, we found out that the conformational changes experienced by GST-Syk after phosphorylation introduced a discrepancy between the conformation of the domains of GST-pSyk and the crystal structures. We then used as a reference the comparison between the EM structure of GST-Syk and the X-

ray structures of isolated domains performed previously [13]. Afterwards, GST-pSyk would be computationally aligned to GST-Syk and the domains annotated by comparison between the two EM structures.

We first fitted the structures of isolated kinase and regulatory domains of Syk into GST-Syk [13] (Supplementary Fig. S1). The fine fitting of the SH2-SH2 region into the EM reconstruction unambiguously mapped the position of the regulatory domains (Supplementary Fig. S1D). Subsequently, the atomic structure of full length Zap-70 [14] was fitted and it was found to corroborate the results of the fitting using the isolated domains of Syk (Fig. 2A). Zap-70 and Syk form a rather flat structure and so, it was obvious that a density protruding at the top of the EM map was not accounted by the kinase domain and it should then correspond to the GST tag. Therefore, these fitting experiments revealed that the GST tag appeared as a density at the back of the flat square-shaped Syk kinase and that the tag was partially responsible for the bulky appearance of the top region in GST-Syk (Fig. 2B, purple colour). Furthermore, the density assigned to the tag occupies a volume compatible with a monomeric GST (Supplementary Fig. S2). In addition, these experiments also allowed the assignment of the regions in the EM reconstruction corresponding to the kinase (Fig. 2B, orange colour) and regulatory (Fig. 2B, green colour) domains. We then aligned the 3D structure of GST-pSyk to the structure of GST-Syk using unbiased computational methods that determined the best relative orientation of one molecule with respect to the other, and the domains of GST-pSyk were then assigned accordingly (Fig. 2C).

3.4 Conformational changes in GST-Syk upon phosphorylation

After alignment of the structures of Syk before and after auto-phosphorylation (GST-Syk and GST-pSyk), significant conformational changes were apparent (Fig. 2B-2C). The relative orientation between the catalytic and regulatory domains in Syk was modified upon auto-phosphorylation. The two SH2 domains of the regulatory region (Fig. 2B, green colour) had changed slightly their relative positions and the region assigned to the inter-SH2 linker seemed to have also moved. Biophysical measurements performed with the SH2 tandem domains have indicated that these domains have sufficient conformational flexibility to bind several target substrates [28]. The conformational rearrangements in the regulatory region of Syk that we observed added further evidence supporting this flexibility and its putative role in the regulation of the protein.

To facilitate the visualization of these changes, a movie was constructed using the GST-Syk and GST-pSyk structures and which shows a simulation of the conformational changes underwent by Syk during its transition from the non-phosphorylated to the phosphorylated state (Supplementary movie S1). The intermediate transitions displayed in this Supplementary movie have no real meaning but they are just a method to help visualize the changes that have taken place between these two structural states determined experimentally, which are shown at the beginning and the end of the movie.

4. Discussion

We have determined the 3D structure of auto-phosphorylated GST-Syk at medium resolution, revealing significant conformational changes in Syk upon phosphorylation. These changes affect mainly to the conformation of the SH2-SH2 cassette and the relative position between this regulatory region and the catalytic domain. Each SH2 domain seems to move with respect to the other SH2 domain and more interestingly, the positioning of the inter-SH2 linker is also displaced. These conformational changes correlate well with current information about the role of the SH2-SH2 region in the regulation of the functions of Syk. For instance, it has been shown recently that Syk autophosphorylation, its phosphorylation by Src and Syk binding to ITAMs through the SH2 domains activate Syk, without synergy among these different stimuli [7].

These authors proposed that this absence of synergy suggested that all these different stimuli activate Syk following similar molecular mechanisms. On the other hand, significant conformational flexibility of the SH2 regulatory region upon binding to its substrates has been described for both Syk and Zap-70 [6,28]. Also, residues locating at the inter-SH2 region have been shown to be essential to maintain the auto-inhibited conformation in Zap-70 [14] and there is evidence for the role of the inter-SH2 linker in the regulation of Syk [2,4]. Together, all these data suggest that conformational transitions in the SH2-SH2 cassette, including the inter-SH2 linker, participate in the control of Syk activation, which correlates with the structural rearrangements that we detect upon auto-phosphorylation.

Based on previous information by other authors and our structures of non-phosphorylated and phosphorylated Syk, we propose a model for the regulation and activation of Syk (Fig. 3A). Syk is maintained in an auto-inhibited conformation by the interactions between the SH2-SH2 regulatory region with the inter-SH2-kinase linker and the catalytic domain that reduce the conformational flexibility required by the kinase domain for catalysis [13]. The structural details of this mechanism have been elucidated thanks to the structure of full-length Zap-70, the other member of the Syk family, at atomic resolution [14]. Phosphorylation of tyrosine residues in Syk and Zap-70 have been shown to contribute to their activation [2,7]. We have now observed that auto-phosphorylation of Syk induces a relative motion of the regulatory region respect to the kinase domain. These rearrangements affect also to the positioning of each of the SH2 modules and the inter-SH2 linker, but the limited resolution of the EM reconstructions preclude any more detailed interpretation of these changes. These motions seem plausible, since the two SH2 domains in Syk and Zap-70 have been found to move either closer or apart depending on their binding to phosphorylated ITAMs [6,28]. We propose that the movements we detect could contribute to the relief of the inhibitory effect of the SH2 region over the catalytic domain (Fig. 3A)[8,14,29].

The EM structure of auto-phosphorylated Syk reported here is consistent with a model of regulation of its catalytic activity that is different from the classical activation step of the Src family of kinases [26,27] (Fig. 3B). Src activation requires large structural rearrangements to relieve the inhibitory effect of the regulatory SH2-SH3 region over the catalytic domain. Several experimental and hypothetical structures suggest that distinct degrees of “opening” of the structure could be required to activate Src [26,27,30] but in all cases, Src activation implies a substantial displacement of one or both regulatory domains with an associated large opening of the structure that relieves the auto-inhibited conformation. In contrast, Syk activation might be achieved with relatively minor conformational changes of the SH2-SH2 region which would be sufficient to alleviate the inhibition over the kinase domain without the need for a large opening in the structure (Fig 3B). Therefore, our observations provide an experimental evidence for the activation model proposed for Zap-70 [14], and suggest that similar mechanisms could be operating in Syk and Zap-70.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Purification and electron microscopy of GST-pSyk. **(A)** Schematic representation of the domain organization of Syk based on the model previously proposed [13]. Regulatory SH2 domains are coloured in green whereas the catalytic kinase domain is shown in orange. These colour codes are maintained throughout the manuscript. **(B)** After auto-phosphorylation, GST-pSyk runs as slower migrating band in an SDS-PAGE (non-phosphorylated (0.5 μ g, lane 1) and phosphorylated (1 μ g, lane 2) GST-Syk). Tyrosine residues located within the inter SH2-kinase region and the C-terminal tail were found to be phosphorylated by mass spectrometry (bottom panel). **(C)** Blue Native electrophoresis of GST-pSyk (lane 3). As a control for mobility of a monomer, GST-pSyk was denatured and run in the same gel (lane 2). Molecular weight

standards are shown in lane 1. **(D)** EM field of negatively stained GST-pSyk with some selected molecules highlighted within circles. Scale bar corresponds to 50 nm. **(E)** One view of the 3D model of GST-pSyk obtained following the random conical tilt method [19]. **(F)** Front and back views of GST-pSyk obtained using angular refinement methods [21]. The 3D structure reveals a large top region facing a smaller domain at the bottom.

Figure 2.

Conformational changes occurring in GST-Syk upon auto-phosphorylation. (A) Three views of the 3D structure of GST-Syk [13] shown as a grey transparent density, where the atomic structure of Zap-70 [14] has been fitted. A region in the EM map not assigned to the kinase (orange colour) and regulatory (green colour) domains after the fitting, was annotated as the GST tag. (B) Front, side and bottom views of GST-Syk and (C) GST-pSyk where the structural regions corresponding to regulatory and kinase domains are coloured in green and orange respectively. The GST tag is coloured in purple. See also supplementary movie S1 on line.

Figure 3.

Model of the regulation of the activation in Syk. **(A)** Cartoon model for the conformational changes induced after auto-phosphorylation of Syk. Inter-SH2 and inter SH2-kinase linkers are depicted as twisted black lanes. **(B)** Comparison between the 3D structures of inhibited and activated Src and Syk. Top panels show the structures of Src [30] (PDB ID 2SRC) and an active form of Src [26](PDB ID 1Y57) solved at atomic resolution by X-ray crystallography. For a better comparison with the structures of Syk solved at medium resolution by EM, the information of these PDB files was computationally filtered to a resolution in the range of that of the Syk structures, and the resulting volumes represented in dark purple and bright orange colour for the inhibited and the active conformation respectively. Bottom panels show the

structures of GST-Syk [13] and GST-pSyk (this work) following the same colour code as in Fig. 2)