Signal transduction: **Clamping down on Src activity**

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Recent high-resolution structures of members of the Src family of protein-tyrosine kinases illustrate how a series of cooperative intramolecular interactions represses the catalytic activity of these kinases, but allows for their rapid activation by a variety of regulatory inputs.

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The protein-tyrosine kinase Src has played a leading role in the history of understanding oncogenes, so the recent description of its three-dimensional structure [1] is a milestone for the fields of cancer research and signal transduction. It is now almost 90 years since Peyton Rous demonstrated that an extract from a tumor of a domestic chicken could induce tumors in other chickens, ushering in the molecular analysis of cancer. The agent responsible, later known as Rous sarcoma virus, became the focus of most of the early studies of retroviral replication and infectivity, and the discovery that the transforming properties of the virus were due to a single gene, termed *src*, that originates from the host genome, confirmed in surprising and dramatic fashion the genetic basis of cancer. The protein encoded by the v-*src* gene was found to be a protein kinase and soon became the prototype of a class of kinases that phosphorylate tyrosine residues of substrate proteins. We now know that protein-tyrosine kinases are intimately involved in signaling in multicellular organisms, either serving directly as membrane-bound receptors or coupled to such receptors in order to integrate responses to a host of growth factors and other extracellular cues.

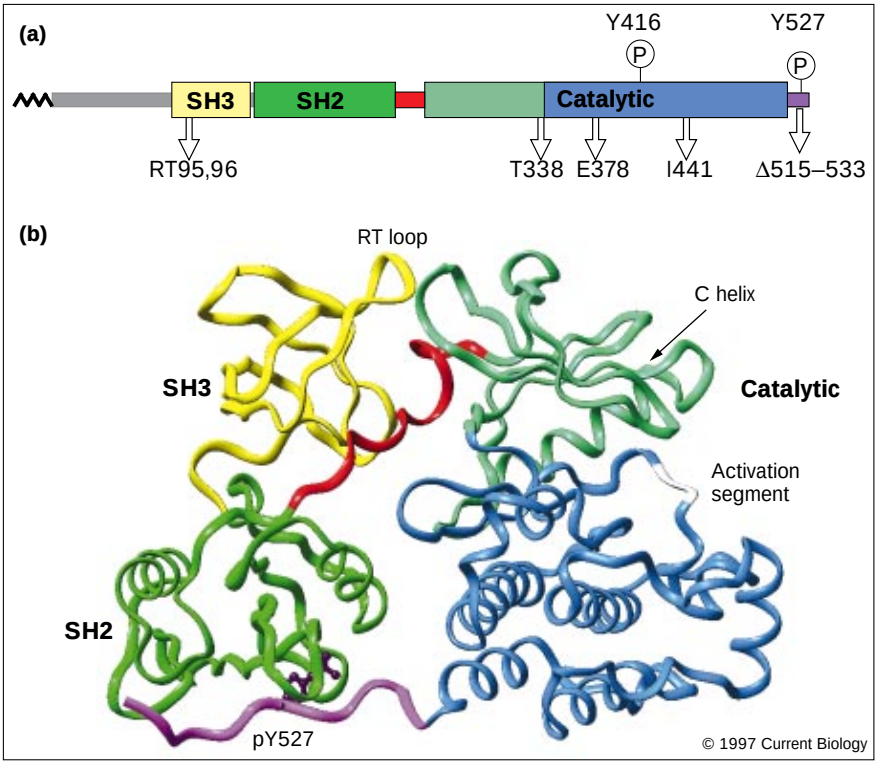
The finding that normal cells make c-Src, the unmutated 'proto-oncogenic' form of the viral kinase, raised a fascinating question: how could a well-behaved and presumably useful kinase be converted into an uncontrolled oncogenic form? Mutagenesis and biochemical analysis by a number of groups led to a model in which the c-Src kinase is held in an inactive conformation by intramolecular interactions that are regulated by tyrosine phosphorylation. The recent solutions of the three-dimensional structures of members of the Src family are entirely consistent with the existing data, and in that sense are unsurprising. But what could never be appreciated by mutagenesis or biochemistry was the elegance of the design of these kinases, or the subtle interplay of domain interactions that reins in the activity of the enzyme yet leaves it poised to spring into action on command.

The Src family of nonreceptor tyrosine kinases consists of nine closely related members, each of which contains an amino-terminal myristoylation site, an amino-terminal segment specific to the particular member, a Src homology 3 (SH3) domain and an SH2 domain, a catalytic domain, and a carboxy-terminal tail containing a regulatory tyrosine phosphorylation site (Fig. 1a) [2]. SH2 and SH3 domains are found in a wide variety of proteins and are small modules that mediate protein–protein interactions: SH2 domains bind specifically to tyrosine-phosphorylated peptides, whereas SH3 domains bind to proline-rich peptides that adopt the polyproline-II (PP-II) helical conformation [3,4]. The Src catalytic domain is quite active when expressed as an individual domain, and repression of its activity is due to the 'regulatory apparatus' consisting of the SH2 domain, the SH3 domain, and the carboxy-terminal tail.

Many studies suggested that phosphorylation of the carboxy-terminal regulatory tyrosine residue (Tyr 527 in chicken c-Src, the prototype of the family) inhibits Src activity *via* an intramolecular interaction with the SH2 domain [2]. Somewhat surprisingly, studies in yeast showed that the SH3 domain was also required for the inhibition of Src by the SH2–tail interaction [5], although the precise mechanism was unclear as there is no obvious proline-rich potential SH3-binding site in the Src protein. In the cell, regulation of Src is thought to be additionally mediated by the actions of other enzymes, such as the tyrosine kinase Csk, which efficiently phosphorylates the carboxy-terminal regulatory site, and phosphatases that can dephosphorylate this site. Mutagenic data have also suggested that the SH2 and SH3 domains can play a positive role in the active kinase: SH2 and SH3 domain mutants of v-Src (which is activated at least in part by elimination of the carboxy-terminal tyrosine phosphorylation site) are crippled to varying extents, being unable to phosphorylate substrates involved in transformation with the efficiency of the 'wild-type' v-Src protein. This probably reflects a role for these domains in localization of the kinase or in substrate binding.

The three-dimensional structures of the 'repressed' forms of c-Src and its relative Hck, comprising the SH2, SH3 and catalytic domains, as well as the phosphorylated carboxy-terminal tail, have been solved recently by X-ray

Figure 1



Structure of human c-Src. (a) Domain structure of Src family kinases. Functional domains are labeled and color-coded to correspond with the three-dimensional structure shown in (b). The locations of the major tyrosine phosphorylation sites (Y416, Y527) are indicated above the domain structure, and the positions of several mutations known to activate c-Src are indicated below. The amino-terminal myristoyl group is represented by a zigzag line. All amino-acid numbering corresponds to the positions in chicken c-Src. (b) Three-dimensional representation of the backbone structure of human c-Src comprising the SH3, SH2 and catalytic domains, and the tyrosine-phosphorylated carboxy-terminal tail (shown as ball-and-stick model). The positions of the structural landmarks that are discussed in text are indicated. The activation segment is not well defined in the structure, and missing residues (410–423) are indicated in white. (The figure was prepared by W. Xu using the program Insight II.)

crystallography [1,6], as has the structure of the active form of the catalytic domain of another Src family member, Lck [7]. The active Lck domain is phosphorylated not on the carboxyl terminus, but instead on a site in the catalytic domain known as the 'activation segment'. Phosphorylation of residues in the corresponding loop correlates with the activation of a wide variety of kinases, including the more distantly related serine/threonine kinases such as protein kinase A (PKA) and cyclin-dependent kinase 2 (Cdk2) [8]. So, what do these structures reveal about the all-important switch between inactive and active forms of kinases?

As expected, in the inactive forms of Src and Hck, the phosphorylated carboxy-terminal tyrosine is bound to the SH2 domain, but somewhat surprisingly the SH2 domain docks onto the 'back' of the catalytic domain (Fig. 1b). The SH3 domain also interacts with the back of the catalytic domain, in this case largely with the linker segment connecting the SH2 domain to the catalytic domain. Unexpectedly, this linker (which contains only one or two proline residues) forms a left-handed PP-II helix and interacts with the normal ligand-binding surface of the SH3 domain. Therefore, in the inactivated form of the kinase, the ligand-binding surfaces of both the SH2 and the SH3 domains are engaged in intramolecular interactions. By contrast, access by either ATP or substrate to the catalytic cleft between the two lobes of the catalytic domain does not appear to be sterically blocked — in fact, the Hck crystals were prepared in the presence of an ATP analog.

The arrangement of the domains in the inactive kinase allows it to be stable and yet at the same time to be subject to rapid activation by triggers such as dephosphorylation of the regulatory tail site. The tail–SH2 interaction is quite weak compared with the binding of the Src SH2 domain to optimal high-affinity ligands [9], so if this interaction were solely responsible for repressing kinase activity, frequent dissociation might be expected to lead to significant spontaneous activation. If, on the other hand, the SH2–tail interaction were stronger, then the phosphorylated tail would almost never be available for dephosphorylation and the kinase could therefore be activated only very slowly if at all. The function of the SH3 domain seems to be to provide a second weak intramolecular interaction that contributes to the overall stability of the inactive conformation. This allows the tail periodically to dissociate from the SH2 domain and to become available for dephosphorylation without fully unleashing the catalytic domain. Of course, if the tail is dephosphorylated, the SH3–linker interaction must be insufficient to stably maintain the inactive conformation.

If the SH3–linker interaction is indeed involved in repressing activity, then interfering with this interaction

should lead to Src activation. Consistent with this suggestion, two residues of the so-called 'RT loop' of the SH3 domain are mutated in v-Src relative to its proto-oncogenic counterpart, and these mutations alone are sufficient to at least partially activate c-Src [10]. The crystal structures reveal that these two residues are involved in specific contacts with the catalytic domain (see Fig. 1b), suggesting that the v-Src mutations would destabilize the SH3–catalytic domain interaction. This issue was directly addressed by a recent study [11] in which purified Hck, a Src family kinase, was mixed with the HIV Nef protein, which is known to bind with high affinity to the SH3 domain of Hck. Addition of Nef increased Hck kinase activity approximately 50-fold, suggesting that high-affinity ligands for the SH3 domain can destabilize the repressed conformation. In fact, Nef was more potent than SH2 ligands in activating the kinase, and it could further activate a kinase lacking the regulatory carboxy-terminal phosphorylation, suggesting that the SH3–catalytic domain interaction is in fact more important than the SH2–tail interaction for maintaining the repressed conformation.

Like a rolled-up armadillo, the inactive form of the kinase presents an inert surface to the environment, with the ligand-binding sites of its SH2 and SH3 domains and the regulatory phosphorylation site tucked inside, and the catalytic domain held in an inactive conformation. The weakness of the individual interactions, however, allows the various regulatory trigger points to become momentarily accessible over time as the structure 'breathes'. The cooperativity of the interactions means that a single activating input — dephosphorylation of the tail, or engagement of the SH2 or SH3 domain by high-affinity ligands — destabilizes the inactive structure and exposes the other trigger points, leading to further activation of the kinase. The increased accessibility to ligand of the SH3 domain of Hck when the SH2–tail interaction is disrupted [11] provides direct evidence for such cooperativity.

But how do these intramolecular interactions regulate the activity of the catalytic domain? Here, a comparison with the active Lck catalytic domain is informative. Protein kinase catalytic domains consist of two lobes, with the catalytic site located in a cleft between them. Comparison of a number of kinase structures reveals that a major difference between inactive and active kinases appears to be the conformation of the activation segment and a helix in the amino-terminal lobe termed the 'C helix' (see Fig. 1b) [8]. Activation usually involves phosphorylation of a site in the activation segment — in the case of Src, Tyr 416 — and this site is indeed phosphorylated in the structure of the active Lck catalytic domain. An important consequence of this phosphorylation in the case of Src family kinases appears to be rotation of the C helix and alignment of a glutamate residue on the helix so that it can interact with Lys295 and assist in the positioning of this residue, which is important in coordinating the phosphate groups of ATP [7]; in the repressed structures of Src and Hck, this glutamate projects into the solvent [1,6]. Remarkably, an identical change in the orientation of the C helix is involved in the activation of Cdk2 by cyclin binding [12].

Of course, this finding merely pushes the question of regulation back one step — how does binding of the SH2 and SH3 domains prevent the phosphorylation of Tyr416 and adoption of the active conformation? Somewhat surprisingly, the structures do not reveal an obvious mechanism for this aspect of Src regulation. Kuriyan and colleagues [6] propose that a hydrophobic residue in the SH2–catalytic domain linker, Trp260, prevents the C helix from assuming its active 'inner' conformation if the SH3 domain is engaged. Eck and colleagues [1] favor a model in which the clamping of the SH2 and SH3 domains onto the back of the catalytic domain in effect paralyzes this domain, preventing the relative movement of the two lobes which is thought to be required for the catalytic cycle.

A number of intriguing questions remain. The first of these concerns the role of phosphorylation of the activation segment. The studies by Miller and colleagues [11] suggest that, for the repressed Hck kinase, phosphorylation of Tyr416 is slow and dependent on the concentration of the kinase, and that it correlates closely with increased kinase activity. This is consistent with a model in which a repressed kinase molecule can, albeit inefficiently, phosphorylate the activation segment of a second molecule and thereby destabilize the inactive conformation of this second molecule. This model implies that activation could be stimulated by high local concentrations of kinase, in agreement with the finding that aggregation triggers the activation of Src family kinases *in vivo* (as in the activation of Lck by clustering of T cell receptors). But what happens if the intramolecular SH2 or SH3 interactions are disrupted? Autophosphorylation of Hck in the presence of Nef is very rapid, suggesting that an intramolecular reaction occurs [11]. Although it remains to be proven, this suggests that one role of the interactions of the SH2 and SH3 domains with the catalytic domain might be to prevent self-activation by intramolecular autophosphorylation.

It is clear, however, that autophosphorylation does not provide the whole story of Src kinase activation — Miller's group [11] showed that a prephosphorylated form of Hck, which was already phosphorylated on the activation segment, could be further activated by Nef. Therefore, when the SH2 and SH3 domains are bound to the catalytic domain, the geometry of the catalytic site or the flexibility of the two lobes must still be compromised even when Tyr416 is phosphorylated. It therefore seems likely that phosphorylation of the catalytic site is a third component

of the interconnected network of cooperative interactions that distinguishes between the active and inactive forms of the kinase — this phosphorylation is insufficient in itself to activate the kinase fully, but it is able to predispose the kinase to further activation by destabilizing the repressed conformation.

The chinks in the armor of the repressed kinase are also apparent in the mutations that occur naturally in v-Src. As a result of decades of passage in a retrovirus under constant selective pressure to cause the most transforming phenotypes, v-Src has evolved to be extremely active. All strains of v-Src have mutations in the carboxy-terminal tail (almost certainly the first activating mutation, occurring at the time the *src* gene was transduced by the retrovirus), the RT loop of the SH3 domain, and the catalytic domain. The SH3 mutations presumably disrupt its interaction with the catalytic domain, as mentioned above; the catalytic domain mutation (Thr338→Ile) lies within the hinge region between the two lobes and presumably destabilizes the inactive conformation of the catalytic site. So, all three components of the inactivating switch have been targeted in v-Src. It is also intriguing that two spontaneous activated mutants of c-Src each have a single amino-acid change in the same region of the carboxy-terminal lobe of the catalytic domain [13]. The location of these mutations suggests that they might destabilize both the binding of the carboxy-terminal tail to the SH2 domain [6] and the inactive conformation of the catalytic domain [1], thereby simultaneously hitting two of the three activation triggers.

Further enzymatic and structural studies should now rapidly lead to a fuller understanding of the role of the individual interactions in triggering the global conformational changes involved in activation of the Src family kinases. It will be important to determine the catalytic activity of different forms of the kinase, which have different phosphorylation states and different intramolecular interactions, as well as the equilibria between these various states in the cell. Mutant forms of the kinase in which the intramolecular interactions are artificially strengthened or weakened by site-directed mutagenesis will help to address these issues. Solving the structures of different phosphorylated forms of the kinase, such as Tyr416–P and Tyr527–P, or structures of the kinase in the presence of activators, such as high-affinity SH2 or SH3 ligands, will also help to pinpoint the precise mechanisms of activation. Because Src family kinases are involved in responses to a variety of important signals, there is hope that these further studies could lead to the design of clinically useful modulators of Src kinase activity.

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