Superti-Furga Team

Tyrosine phosphorylation and protein-protein interactions

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SH2 domains and tyrosine phosphorylation are major biochemical hallmarks of multicellular organisms and their advent may have been instrumental to metazoan evolution. Thus, tyrosine phosphorylation is involved in a great variety of processes related to cell-cell communication and its dysfunction is associated with many important human diseases. The network of tyrosine phosphorylation pathways in the vertebrate cell is very complex and still largely uncharted. We are studying the activity and regulation of two prototypic tyrosine kinases, c-Src and c-Abl, with the idea that in-depth understanding of these "building blocks" may serve as key to the logic of complex tyrosine phosphorylation networks. c-Src and c-Abl are ubiquitous in higher organisms, have been implicated in a variety of cellular functions in the cell cytoplasm and in the nucleus and have oncogenic counterparts. Our studies show that these proteins are intimately associated with protein-protein interactions. On one hand their activity is regulated by an exquisite set of intramolecular associations. On the other hand their catalytic activity itself can determine the ability to interact with other proteins. By virtue of these properties, Src and Abl function as regulatory "assembler" of protein interactions, rather than diffusable phosphorylation units. We believe that this reflects the essence of tyrosine phosphorylation circuits: dynamic networks of protein-protein interactions involved in signaling. We have started a phosphoproteomic approach to begin charting tyrosine phosphorylation events in the nucleus.

Regulation mechanics of the Src family of tyrosine kinases

c-Src is inhibited by phosphorylation of Tyr 527 at the C-terminal end by the Csk tyrosine kinase. We have studied the structural components necessary for activity and regulation based on the crystal structure of Src. In the structure, the SH3 and SH2 domains of Src are located in the "back" of the catalytic domain (opposite of the substrate and ATP-binding site, see Figure 1). The "tail", purple in the figure, containing the phosphorylated tyrosine residue (Tyr527) reaches over to the SH2 domain (yellow) and binds to it. The SH3 domain (green) binds to the region linking the SH2 domain to the catalytic domain (SH2-CD linker, red) in a three-partite sandwich involving the small lobe of the catalytic domain (blue). In this conformation, inactivity is multiple: the catalytic domain is repressed by misplacement of residues important for catalysis and by a conformation of the activation loop (orange) that is hindering substrate access, and the SH2 and SH3 are unavailable for interactions with other proteins.

Bidirectional regulation of Src

(Stefania Gonfloni, Jana Kretzschmar)

In the inactive state, the critical αC helix in the catalytic domain is misplaced and Tyr 416 in the activation loop (in orange in Figure 1) is unphosphorylated, while the SH2 and SH3 domains are unavailable for interactions with other proteins. We found that phosphorylation of the activation loop or mutation of the loop preceding the αC helix, activates Src while also increasing the accessibility of the SH3 domain for ligands. A large interaction surface of the αC helix with the activation loop is a central component of this regulatory system. Our data suggest a bidirectional regulation mechanism, in which not only the regulatory domains inhibit Src activity, but the activity itself controls the availability of the regulatory domains and therefore the interaction potential of the molecule. By this mechanism, Src family kinases can be activated by proteins phosphorylating or changing the conformation of the catalytic domain. Once active, Src family kinases become less prone to regulation, implying a positive feedback loop on their activity.

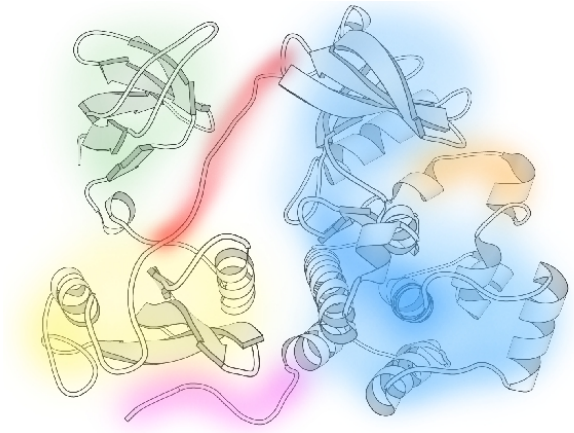


Figure 1. *Three-dimensional structure of human c-Src. (See text for details.)*

A novel interaction mode for the SH2 domain

(John R. Engen, Christophe Cans, Jana Kretzschmar)

In the structure of regulated inactive c-Src, there is large interface between the regulatory SH3, SH2 domains together with the SH2-CD linker (as white worm in Figure 2) on one side and the catalytic domain (as purple worm in Figure 2), on the other side. Investigating the interaction surfaces within Src involved in regulation, until now most attention has been devoted to the SH3 domain because the SH2 domain was thought to "simply" bind to the C-terminal phosphorylated tail of Src and have no additional roles. To test the hypothesis that the SH2 may have additional roles in the regulation of Src, we tested the importance of the "lower" part of the interface shown in Figure 2. We mutated several residues at the interface between the Src SH2 domain and the "back" of the large lobe of the catalytic domain. In the Src-family of proteins, these residues are highly conserved charged residues that could form salt bridges. When the interacting residues are mutated to alanine, Src becomes completely deregulated, showing that indeed, these interactions are crucial for regulation.

In Abl and related proteins, that lack regulation by engagement of a C-terminal tyrosine with the SH2, the residues in the positions corresponding to the SH2-catalytic domain interface are aromatic. When combined with a third residue at the very end of the catalytic domain, the three aromatic residues are in the correct position to form pi-stacking interactions with one another. When these SH2-catalytic domain interface residues were mutated in c-Abl, the protein became deregulated like in the corresponding mutants of Src. All together, our results suggest that there is a regulatory role for the αA-helix of the SH2 domain in addition to its well established role in binding phosphorylated tyrosine. We propose that this unexpected, "atypical" protein-protein interaction property of the SH2 domain may be important in other proteins as well. The results also support the idea that to keep Src and Abl in a regulated from, there are several small contributes to regulation, any of which can be triggered to activate the enzyme.

Regulation of c-Abl

(Helma Pluk, Karel Dorey, John R. Engen, Jana Kretzschmar)

For many years, researchers have been looking for a protein inhibiting Abl activity that interacted with the Abl SH3 domain. We have found some years ago that the SH3 domain of Abl inhibits Abl activity intramolecularly (as in Src). Using a structural model based on the new Abl structure, the "old" structure of regulated Src and the structures of the Abl SH3 and SH2 domains, we have undergone a novel round of mutagenesis, functional and biochemical experiments. While these experiments are not completed yet, they are entirely consistent with the intramolecular regulation model we had previously proposed.

The role of the activation loop in c-Abl

(Karel Dorey, John R. Engen, Jana Kretzschmar, in collaboration with Thomas Schindler and John Kuriyan, Rockefeller University)

Constitutively active forms of Abl, like the oncogenic BCR-Abl and v-Abl fusion proteins, are constitutively tyrosine phosphorylated. The same is true for deregulated forms of c-Abl, like point mutations in the SH3 domain or in the SH2-CD linker. This suggests that Abl activity and tyrosine phosphorylation of Abl proteins go hand in hand. We tested, whether also the endogenous cellular form of the enzyme could become tyrosine phosphorylated. We identified treatments that induced tyrosine phosphorylation of c-Abl and a correlating increase in catalytic activity. Tyrosine phosphorylation of the oncogenic forms of Abl occurs on a tyrosine residue in the activation loop. Based on the new structure of the Abl catalytic domain bound to the STI-571 inhibitor obtained by the laboratory of John Kuriyan, we performed a functional study to understand the role of the activation loop in the regulation of c-Abl. In the structure, the unphosphorylated activation loop mimics a peptide substrate while Tyr412 points inward and interferes with catalysis. We mutated residues involved in stabilizing the activation loop and positioning Tyr412. These mutations resulted in tyrosine phosphorylation and activation of c-Abl. Tyr412 in the activation loop became phosphorylated in activated c-Abl as measured by mass spectrometry. Phosphorylation occurred autocatalytically by a *trans-*

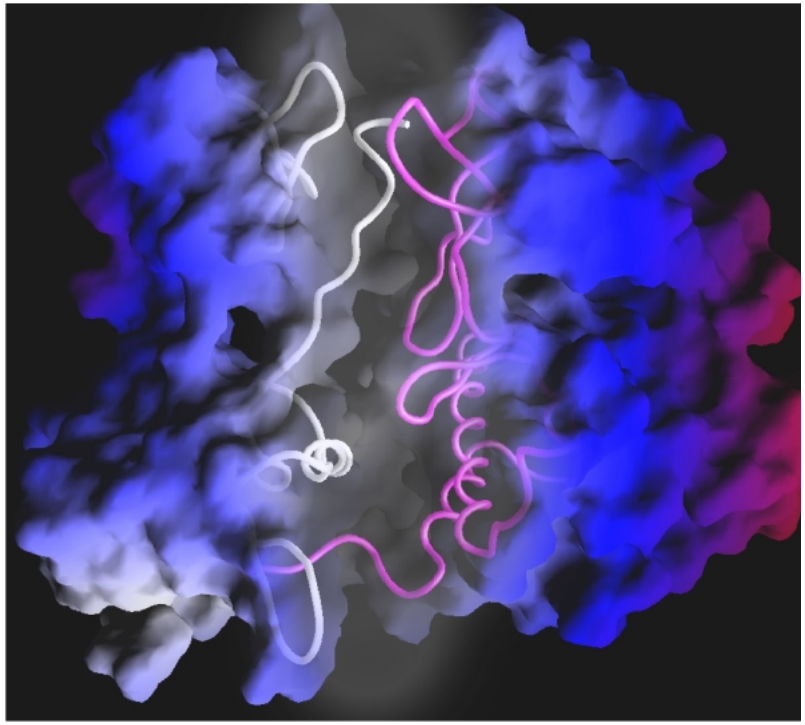
mechanism and caused activation of otherwise inactive c-Abl, suggesting a positive feedback loop on c-Abl activity. Tyr412 itself was required for efficient catalytic activity and signaling function. Tyr412 and the activation loop form the central regulation mechanism of c-Abl and stabilize the inactive or the active conformation of the enzyme in a phosphorylation-dependent manner.

A nuclear tyrosine phosphorylation circuit: c-Jun as an activator and substrate of c-Abl and JNK

(Raffaella Mangano, Filippo Cipriani, Jana Kretzschmar)

The c-Abl tyrosine kinase is among the few tyrosine kinases to localize both to the nucleus and cytoplasm of cells. In dependence on its localization, both positive and negative effects of growth and apoptosis have been proposed. We found that the c-Jun transcription factor can activate c-Abl in the nucleus (in collaboration with Dirk Bohmann, see last year's report). A proline-rich region in c-Jun binds the c-Abl SH3 domain and is required for initial activation. c-Jun becomes a nuclear substrate of Abl. After c-Jun is phosphorylated by Abl on tyrosine 170, the two proteins interact via the SH2 domain of Abl. Surprisingly accumulation of c-Jun protein in the nucleus resulted in Abl activation and activation of the JNK stress-activated protein kinase. Experiments with cells derived from c-Jun knock-out or c-Abl/Arg double knock-out fibroblasts showed that TPA treatment in the presence of serum led to a slow but sustained activation of JNK activity that was dependent on c-Jun and c-Abl/Arg. This mechanism of tuning nuclear JNK activity could be involved in integrating growth and stress stimuli to cellular fate decisions and represents a case of intranuclear tyrosine phosphorylation.

Figure 2. *Interface between the SH3/SH2/linker and the catalytic domain of Src. See text for details.*



A proteomic approach to nuclear tyrosine phosphorylation

(Christophe Cans in collaboration with Gitte Neubauer and Matthias Wilm)

Tyrosine phosphorylation is usually associated with cytoplasmic events. Yet over the years many reports have accumulated on tyrosine phosphorylation of individual molecules in the nucleus and several tyrosine kinases and phosphatases have been found to be at least partially nuclear. The question arises, whether nuclear tyrosine phosphorylation represents a collection of loose ends of events originating in the cytoplasm or if there may be intranuclear signaling circuits relying on tyrosine phosphorylation to regulate specific processes. Our recent discovery of a mechanism causing nuclear tyrosine phosphorylation (see above) has prompted us to review the cumulative evidence for nuclear tyrosine phosphorylation pathways and their possible role and to start a systematic analysis of nuclear tyrosine phosphorylation.

To this end we have started to treat a variety of human and mouse cell lines with vanadate or with other agents known to elicit tyrosine phosphorylation. We have prepared nuclear extracts and purified tyrosine-phosphorylated proteins using a column to which antibodies against phosphotyrosine were coupled.

After purification, the target proteins are identified by mass spectrometric analysis.

In a complementary approach we look specifically at nuclear substrates of Abl. c-Abl protein shuttles between the nucleus and the cytoplasm in mammalian cells. Jean Wang's and Rick Van Etten's laboratories have created mutations in Abl that restrict Abl localization to either the cytoplasm or the nucleus. During our studies on Abl regulation, we have generated alleles of Abl that are highly active, but have otherwise preserved the structure of the molecule. We have combined this activating point mutations with mutations affecting the subcellular localization of Abl. Using efficient transfection procedures or inducible expression systems we are now looking systematically at nuclear proteins that are phosphorylated by Abl. We hope that this will allow us to understand the role of Abl in the nucleus.

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