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A CASE STUDY OF PROLINE ISOMERIZATION IN CELL SIGNALING

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1. ABSTRACT

Protein-mediated interactions and enzymatic function provide the foundation upon which cellular signaling cascades control all of the activities of a cell. Post-translational modifications such as phosphorylation or ubiquitiation are well known means for modulating protein activity within the cell. These chemical modifications create new recognition motifs on proteins or shift conformational preferences such that protein catalytic and binding functions are altered in response to external stimuli. Moreover, detection of such modifications is often straightforward by conventional biochemical methods leading investigators toward mechanistic models of cell signaling involving post-translational modifications such as phosphorylation/dephosphorylation. While there is little doubt that such modifications play a significant role in transmission of information throughout the cell, there are certainly other mechanisms at work that are not as well understood at this time. Of particular interest in the context of this review is the intrinsic conformational switch afforded to a polypeptide by peptidyl prolyl cis/trans isomerization. Proline isomerization is emerging as a critical component of certain cell signaling cascades. In addition to serving as a conformational switch that enables a protein to adopt functionally distinct states, proline isomerization may serve as a recognition element for the ubiquitous peptidyl prolyl isomerases. This overview takes a close look at one particular signaling protein, the T cell specific tyrosine kinase Itk, and examines the role of proline isomerization and the peptidyl prolyl isomerase cyclophilin A in mediating Itk function following T cell receptor engagement.

2. THE PEPTIDYL PROLYL ISOMERASES AND THE NON-RECEPTOR TYROSINE KINASES: BALANCING FUNCTIONS IN T CELL SIGNALING

2.1. Immunosuppressive natural products cyclosporin A and FK506 target the ubiquitous peptidyl prolyl isomerases

Cell signaling relies on a complex array of protein-mediated interactions that ultimately determine cell fate. Precise control of both protein-mediated interactions and enzymatic activity is a critical component of signal transduction and therefore cellular homeostasis. Exogenous factors such as small molecule ligands can alter regulation of protein activity and have profound effects on cellular behavior. The natural products cyclosporin A (CsA) and FK506 are well-studied examples of small molecules capable of interfering with a cellular signaling pathway (1). These drugs have found extensive use as immunosuppressive agents by specifically targeting the peptidyl prolyl isomerases (PPIases) of the cyclophilin (Cyp) and FK506 binding protein (FKBP) families (2-4). The resulting binary CsA-Cyp or FK506-FKBP complex binds to and inhibits calcineurin (5, 6), the serine/threonine phosphatase responsible for dephosphorylation and subsequent nuclear

translocation of the IL-2 transcription factor, nuclear factor of activated T-cells (7). In this manner, T cell activation is halted and the immune response is suspended. In addition to the already mentioned cyclophilins and FKBPs, a third family of peptidyl prolyl isomerases, the parvulins, fulfill critical cellular functions. The most well studied member is Pin1 (8), which, unlike the cyclophlins and FKBP family, functions in a phosphorylation-dependent manner and is essential for cell growth (9). Several recent reviews of Pin1 isomerase function provide an overview of relevant work in this area (10-12).

As their names suggest, characterization of the cyclophilins and FKBPs was accelerated by virtue of their affinity for the immunsuppressive agents, CsA and FK506. In an unexpected turn, the mode of action of CsA and FK506 in suppressing the immune response is not related to the fact that the isomerase activities of Cyp and FKBP are inhibited by binding to CsA and FK506, respectively. Thus, elucidation of the endogenous functions of the ubiquitous peptidyl prolyl isomerases has lagged behind our understanding of these proteins as the intracellular receptors for CsA and FK506. Adding to the complexity of the problem is the fact that multiple isoforms of the PPIases exist. For example, CypA, CypB, CypC and CypD all share sequence homology yet differ with respect to subcellular localization and therefore functional significance (13). Whatever the precise role of each isoform, all exhibit peptidyl-prolyl cis/trans isomerase activity and this activity is inhibited by cyclosporin.

2.2. Isomerases: from folding chaperone to cell signaling molecule

Isomerase activity promotes the rate of interconversion between cis and trans prolyl imide bond conformers in a proline containing peptide as was first demonstrated using a linked enzymatic assay that relies on the ability of chymotrypsin to preferentially cleave the trans imide bond in a model peptide substrate (14). This assay not only allows simple and rapid detection of isomerase activity but also exploits a prototypical example of conformer specific substrate recognition by a protease (15, 16). Isomerase activity has long been thought to influence the kinetics of protein folding (17) as prolyl isomerization is a slow step in protein denaturation and renaturation (18). A role for PPIase activity in protein folding was first demonstrated in experiments that showed a delay in collagen triple helix formation in chick embryo tendon fibroblasts in the presence of CsA (19). Direct evidence for the involvement of PPIase activity (both cyclophilin and FKBP types enzymes) in folding of two bacterial luciferases was then provided in the context of a cell free translation system (20). Thus, these early examples set the stage for establishing the PPIases as folding chaperones and more recent work has expanded the repertoire of the PPIases beyond that of folding catalyst (21).

It has long been postulated that proline isomerization and the isomerases that catalyze this conformational exchange event may serve as molecular switches that control biological processes (22). Indeed, the cyclophilins alone have been implicated in processes as diverse as trafficking, signal transduction, regulation of the cell cycle, differentiation, transcriptional control, stabilization of multi-protein complexes and have been shown to have antioxidant activity (23-33). Clearly, the peptidyl prolyl isomerases carry out numerous biological functions and it is likely that our understanding of the functional scope of these enzymes will only increase with time. The remainder of this review will focus on the functional role of proline isomerization itself and cyclophilin A function in controlling the catalytic activity of a T cell specific non-receptor tyrosine kinase.

2.3. Tyrosine kinases: transmitting the signal by phosphorylation

Mechanistically, the protein tyrosine kinases play key roles in transmission of cellular signals. In concert with the protein tyrosine phosphatases (34), tyrosine kinases directly modulate intracellular levels of phosphotyrosine. Thus, proper regulation of these catalytic activities is absolutely essential to maintain normal cellular processes as aberrant phosphorylation can either halt or trigger downstream signals drastically effecting cell fate. (35). The prototypical Src tyrosine kinase family (36) consists of nine members (Src, Yes, Fgr, Yrk, Fyn, Lyn, Hck, Lck and Blk) and is the best understood in terms of the mechanism by which catalytic activity is controlled (37, 38).

The three dimensional structures of two Src family kinases in their inactive state, Src and Hck (39, 40), along with the crystal structure of an active kinase domain, Lck (41), capped decades of biochemical and genetic work and provide great insight into the molecular details of Src kinase regulation (42). Namely, two key regulatory interactions involving the non-catalytic Src homology 2 (SH2) and Src homology 3 (SH3) domains of Src serve to downregulate kinase activity. More specifically, phosphorylation of a tyrosine residue in the carboxy-terminal tail of Src by another tyrosine kinase, Csk (43, 44), leads to Src inactivation via intramolecular contacts between the resulting phosphotyrosine (Tyr 527) and Src's own SH2 domain. A second intramolecular contact is formed between the Src SH3 domain and the linker sequence between the SH2 and kinase domains. Together, these interactions serve to restrict access to both the SH2 and SH3 domains and, by influencing the conformational properties of the neighboring kinase domain, render the catalytic domain unable to carry out its chemistry. The regulatory tyrosine residue, in particular, is a hallmark of the Src family kinases and its absence in other protein tyrosine kinase families raises interesting questions regarding alternative modes of regulation.

After the Src kinase family, the Tec family kinases comprise the second largest class of non-receptor protein tyrosine kinases (45-47). Inspection of the primary amino acid sequences of each of the five Tec kinases (Itk, Btk, Tec, Rlk and Bmx) reveals the absence of the carboxy terminal regulatory tail and thus the absence of the regulatory tyrosine residue as found in the Src family (48). This small yet significant difference in sequence between the two families demands that the regulatory mechanism

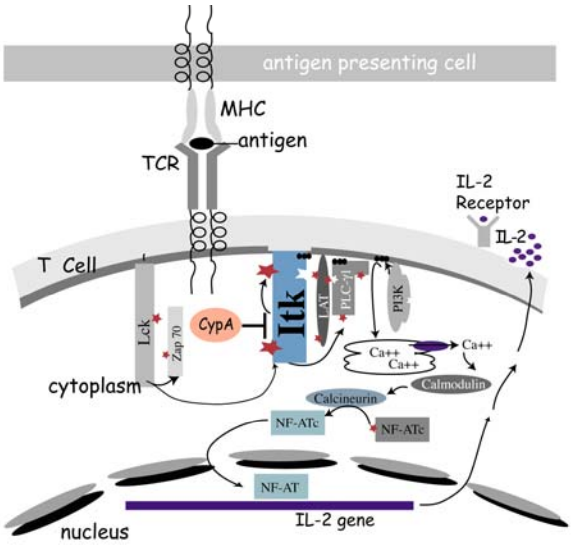

Figure 1. T cell stimulation following MCH/antigen recognition by the T cell receptor (TCR). Various signaling proteins involved in T cell activation are shown. Cyclophilin A is shown as a negative regulator of Itk. Phosphorylation sites are indicated by red stars.

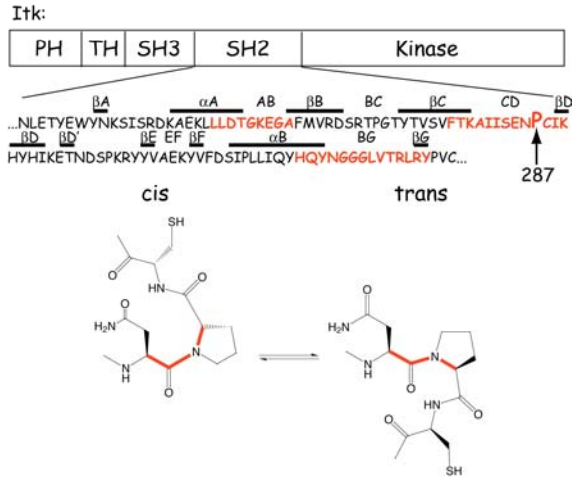

Figure 2. Domain structure of Itk. The protein consists of the catalytic domain (labeled Kinase), Src homology 2 (SH2) and Src homology 3 (SH3) domains, a region of conservation among the Tec kinases termed the Tec homology (TH) domain and a Pleckstrin homology (PH) domain. The primary amino acid sequence of the SH2 domain is shown with Pro 287 in large type. Secondary structural elements are indicated above the sequence. The residues that give rise to doubled resonances in NMR spectra of the Itk SH2 domain are indicated in bold red type. The chemical structures of the cis and trans imide bond conformations of a prolyl containing peptide are shown. The peptide bond that isomerizes is highlighted in red.

for the Tec kinases is at least in part distinct from that of the Src kinases. For members of the Tec family kinases, the nature of this regulatory switch is only now coming into focus (28, 49-54) and appears quite distinct from that of the well-studied Src kinases (40, 55). Of particular interest to this overview is the fact that the regulatory apparatus for the T cell specific non-receptor Tec family kinase, Itk, involves the peptidyl-prolyl isomerase CypA. Itk (Interleukin-2 tyrosine kinase) is the Tec family kinase that is required for intracellular signaling immediately following T cell receptor (TCR) crosslinking (Figure 1) (56, 57).

2.4. A regulatory role for CypA in cell signaling: control over the Itk kinase

Experiments in Jurkat T cells suggest that the isomerase activity of CypA negatively regulates the catalytic activity of Itk (28). Subsequent work in primary T cells lacking the CypA gene showed increased Itk function (58). Moreover, the down-regulation of Itk by CypA is reversed by cyclosporin A, implicating the CypA active site in regulation of this kinase (28). Together, these findings indicate that, in addition to the well-known immunosuppressive effects, inhibition of CypA by CsA has a direct effect on tyrosine kinase regulation. Thus, in the absence of exogenous small ligands such as CsA, cyclophilin serves to down regulate signals originating from the T cell receptor by limiting Itk catalytic activity (Figure 1). The precise mechanism by which CypA regulates Itk kinase activity has not yet been elucidated to the same extent as that of the Src regulatory apparatus. Regardless, the Itk-CypA regulatory interaction represents a novel mode of tyrosine kinase regulation and clearly places CypA among the signaling molecules that control the downstream effects of TCR stimulation.

2.5. The CypA target, Itk, is a conformationally heterogeneous T cell protein

The regulatory effect of CypA on Itk catalytic activity was discovered by virtue of a conformationally heterogeneous proline residue within a non-catalytic domain of Itk. The SH2 domain of Itk contains a single proline residue (Pro 287) that adopts both the cis and trans imide bond conformations in solution (Figure 2) (28, 59). This conformational exchange event appears to play a significant role in Itk mediated signaling. Most notably, a single point mutation at position 287 within full length Itk leads to increased Itk signaling in primary T cells (58). Specifically, replacement of the conformationally heterogeneous proline residue with glycine (an amino acid that exclusively favors the trans peptide bond conformation) releases Itk from regulation by CypA. Thus, the dynamic exchange of Pro 287 between two conformations is a critical component of the regulatory apparatus of Itk. Additionally, structural studies show that Pro 287 serves as a fulcrum about which the Itk SH2 domain switches between two conformers that bind distinct signaling partners (59, 60). The remainder of this review will focus on the structural features of this proline-switch, how the two Itk substates bind distinct ligands and finally we will present a model for how proline isomerization may regulate kinase activity during T cell signaling. The work carried out on Itk to date suggests that, in a manner similar to protein phosphorylation, proline isomerization has the potential to serve as a general molecular switch that

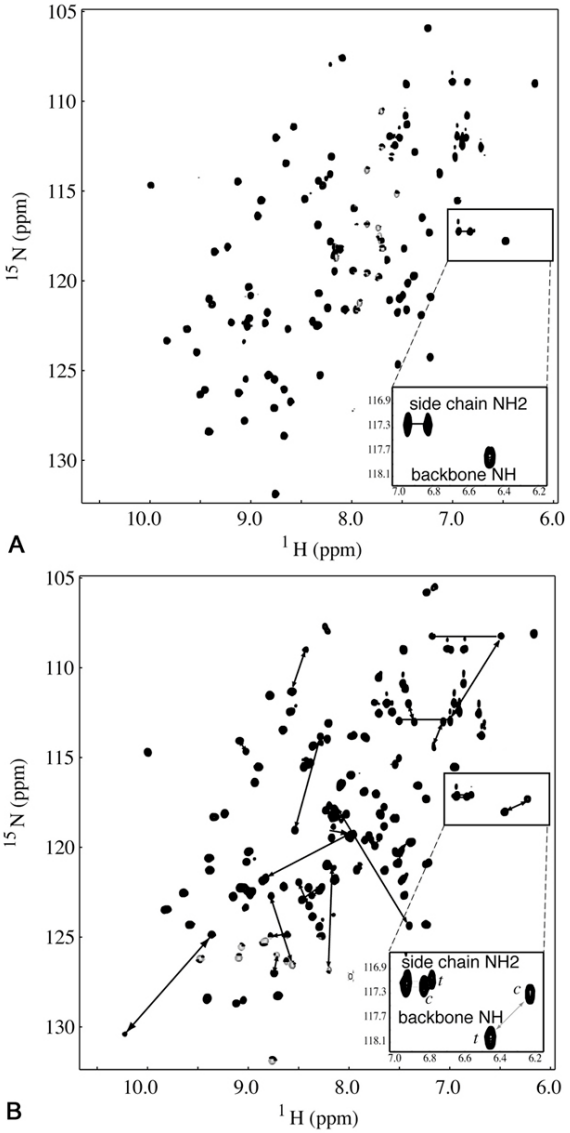

Figure 3. A. Two-dimensional heteronuclear single quantum correlation (HSQC) spectrum of an ^{15}N labeled protein that adopts a single low energy conformation in solution. Every NH group in the protein gives rise to a crosspeak that resonates at a specific proton and nitrogen frequency. A region of the spectrum containing an asparagine (NH_2) side chain resonance and a backbone amide resonance is expanded. B. The same HSQC experiment for a conformationally heterogenous protein reveals doubled resonances due to peptidyl prolyl cis/trans isomerization for 35 of 109 amino acids. The doubled resonances are indicated with a line between the two peaks corresponding to the cis and trans imide bond containing conformers. The asparagine (NH_2) side chain resonance and backbone amide resonance are expanded as in (a) and each give rise to two separate resonances labeled *t* (trans) and *c* (cis). Note: the protein sample used to generate the spectra in this figure are two forms of the Itk SH2 domain. The sample in (b) is wild type Itk SH2 and the protein used to generate the spectrum in (a) is identical except that it contains a single point mutation that removes the conformationally heterogenous proline residue and replaces it with glycine.

controls the function of a multitude of signaling proteins. Furthermore, such conformational switches may be regulated by the action of the peptidyl prolyl isomerases.

3. PROLINE ISOMERIZATION IN A CELL SIGNALING PROTEIN

3.1. Proline isomerization probed by nuclear magnetic resonance spectroscopy

Proline is structurally unique among the naturally occurring amino acids in that both the cis and trans peptide bond conformations are significantly populated at physiological temperature (61, 62). This residue lends the polypeptide backbone the ability to populate structurally and dynamically distinct conformational states. Moreover, the cis and trans imide bond conformers are separated by an interconversion activation energy of 14-24 kcal/mol leading to a slow rate of exchange between conformers (63). Data for short proline containing peptides indicate that prolyl isomerization proceeds via a one step mechanism involving a planar transition state (64, 65). Proline cis/trans isomerization is a well-studied phenomenon in the context of short flexible peptides but is significantly less studied within the confines of the folded, stable tertiary structure of native proteins. Indeed, while a number of protein structures have been solved to date that exhibit proline cis/trans isomerization (66); most proline residues within folded proteins populate one imide bond conformer or the other and not both. A recent review of 'native-state' proline isomerization reveals some shared features of folded proteins that harbor conformationally heterogeneous prolines (67). Proline switches in folded proteins may, in fact, be accompanied by specific sequence and structural features and understanding these hallmarks could eventually facilitate identification of native state proline isomerization from primary amino acid sequence. At this time however, the identification of conformationally heterogeneous prolines relies on experimental techniques such as nuclear magnetic resonance (NMR) spectroscopy.

NMR spectroscopy is an extremely powerful tool for the interrogation of biomolecular structure and dynamics and is the most direct method for detection of prolyl isomerization in native proteins and peptides. With respect to the NMR chemical shift timescale, peptidyl prolyl cis/trans isomerization is slow, giving rise to two observable resonance frequencies per nuclear spin in the proximity of the isomerizing bond (Figure 3). The slow kinetics make possible the identification of folded proteins that harbor a conformationally heterogeneous proline by simple inspection of NMR data. For example, a ^{1}H-^{15}N correlation (HSQC) spectrum (68) of a protein that adopts only a single low energy structure will be comprised of a single crosspeak per NH functional group in the protein (backbone amide groups plus sidechain NH groups) (Figure 3a). In contrast, a corresponding spectrum of a conformationally heterogeneous protein molecule undergoing slow exchange between conformations will contain 'extra' crosspeaks that are attributable to the additional conformational states (Figure 3b). Thus, an accounting of the number of crosspeaks in the HSQC spectrum reveals the presence of multiple conformations in

solution. Proline isomerization can then be determined as the source of conformational heterogeneity if the 'extra' crosspeaks are abolished in a spectrum of a mutant protein lacking proline (Figure 3). While this approach requires significant quantities of isotopically labeled protein and may therefore not be well suited to high throughput screening approaches, it can be carried out prior to resonance assignment and full structure determination and therefore requires very little investment in NMR data analysis.

In this manner, preliminary NMR analysis of the purified ^{15}N-labeled Itk SH2 domain revealed a total of 144 crosspeaks; a number inconsistent with the expected 109 crosspeaks for the SH2 sequence (Figs. 2 and 3) (28). Spectral dependence on temperature confirmed the presence of two species in solution interconverting at a rate that is slow with respect to the chemical shift timescale. To specifically evaluate the putative contribution of proline isomerization to the observed conformational heterogeneity, mutational analysis of each proline residue in the wildtype sequence was undertaken. The HSQC spectrum of the domain containing the Pro287Gly mutation confirmed that the conformational heterogeneity within the Itk SH2 domain was due exclusively to proline isomerization about the 286-287 imide bond (28). For the Pro287Gly SH2 domain, the number of ^{1}H-^{15}N crosspeaks correlated exactly with the expected number of NH groups in the conformationally homogeneous SH2 domain indicating that, unlike wild type protein, the mutant protein adopts only a single low energy structure in solution (Figure 3). Thus, simple inspection of NMR datasets acquired for wild type and mutant protein revealed a proline-driven conformational exchange event within the Itk SH2 domain.

A recent structural study of the α-hemoglobin chaperone protein (AHSP) provides a strikingly similar view of proline induced conformational heterogeneity in a folded protein (69). In that case, two equally populated prolyl imide bond containing conformers give rise to a large number of 'extra' signals in NMR spectra of the wild type AHSP protein. As for Itk SH2, mutation of a single proline residue (Pro 30 of AHSP) to alanine led to a significant reduction in the number of observed NMR peaks. Mutation to a residue that is restricted to the trans conformation results in a conformationally homogeneous protein structure. As discussed in detail for Itk below, proline isomerization within AHSP also results in significant structural differences between the cis and trans forms which are exploited by α-hemoglobin (the binding partner of AHSP) resulting in conformer specific ligand recognition (69).

3.2. Structural consequences of native state proline isomerization

The structural perturbations arising from proline isomerization within the Itk SH2 domain are pronounced and broadly affect the NMR chemical shifts of the resonances of the SH2 domain. Approximately one third of the residues within the domain give rise to doubled resonances and are therefore affected by isomerization about the single 286-287 imide bond (Figure 2). Two factors, the chemical shift dispersion between resonances corresponding to the cis and trans conformers and the relatively high population of each conformer (60% trans and 40% cis), allowed determination of the three dimensional structures of the SH2 domain in its two forms using a single NMR dataset (59). Both the cis and trans conformers adopt the typical SH2 fold consisting of a central, three strand antiparallel β-sheet, flanked by two alpha helices (Fig 4a). The SH2 residues in slow exchange between conformers are located on the A helix (αA), the AB-loop, the CD-loop (including Pro 287), the B helix (αB) and the BG loop (Figure 2). These structural elements are separated in amino acid sequence, but form a contiguous surface over approximately one-third of the SH2 domain (Figure 4b).

The most striking structural difference between the cis and trans forms of the Itk SH2 domain is a bend at Pro 287 itself and at the corresponding cross-strand residue, Lys 280. These two residues are located at the top of the central β-sheet in the SH2 domain and are the bookends for the CD loop. The bend about the Pro 287/Lys 280 hinge rotates the CD loop by approximately 60° between the cis and trans conformers (Figure 4c). It is notable that the CD loop adopts different dynamic behavior in the trans versus cis form of the domain as is apparent in both independent dynamics measurements (59) and in the dispersion of the overlayed NMR structures (Figure 4c). Specifically, the CD loop in the cis imide bond-containing SH2 conformer is significantly more ordered than in the trans form. Of particular interest are the precise molecular contacts that stabilize the cis proline conformer. The CD loop of the Itk SH2 domain contains a number of hydrophobic residues (Ala281, Ile282, Ile283, Ile289) and the structures of the two forms of the SH2 domain reveals that these side chains pack together more efficiently in the cis structure than in the corresponding trans structure (Figure 4c). Thus it is plausible that the cis imide bond at position 286-287 is stabilized by interactions between specific hydrophobic side chains in the surrounding primary amino acid sequence. Moreover, Pro 287 itself must be in a location that is amenable to conformational exchange and in the structures of both SH2 conformers it is clear that this residue is in a flexible loop region rather than in a region of stable secondary structure. The conformationally dynamic Itk SH2 domain (59), the α-hemoglobin chaperone AHSP (69) and the other proteins for which dual structure determination has been possible to date (67, 70-72), are emerging as ideal model systems to understand the molecular determinants of native state proline isomerization and may pave the way to recognizing and characterizing this type of proline switch in unrelated protein sequences.

3.3. Proline isomerization imparts dual ligand binding capability

To shed additional light on the role of proline isomerization in controlling Itk mediated T cell signaling, the function of the SH2 domain as a protein binding module must be considered. Like all SH2 domains studied to date, Itk SH2 recognizes phosphotyrosine-containing sequences via two adjacent binding pockets: the 'pY'

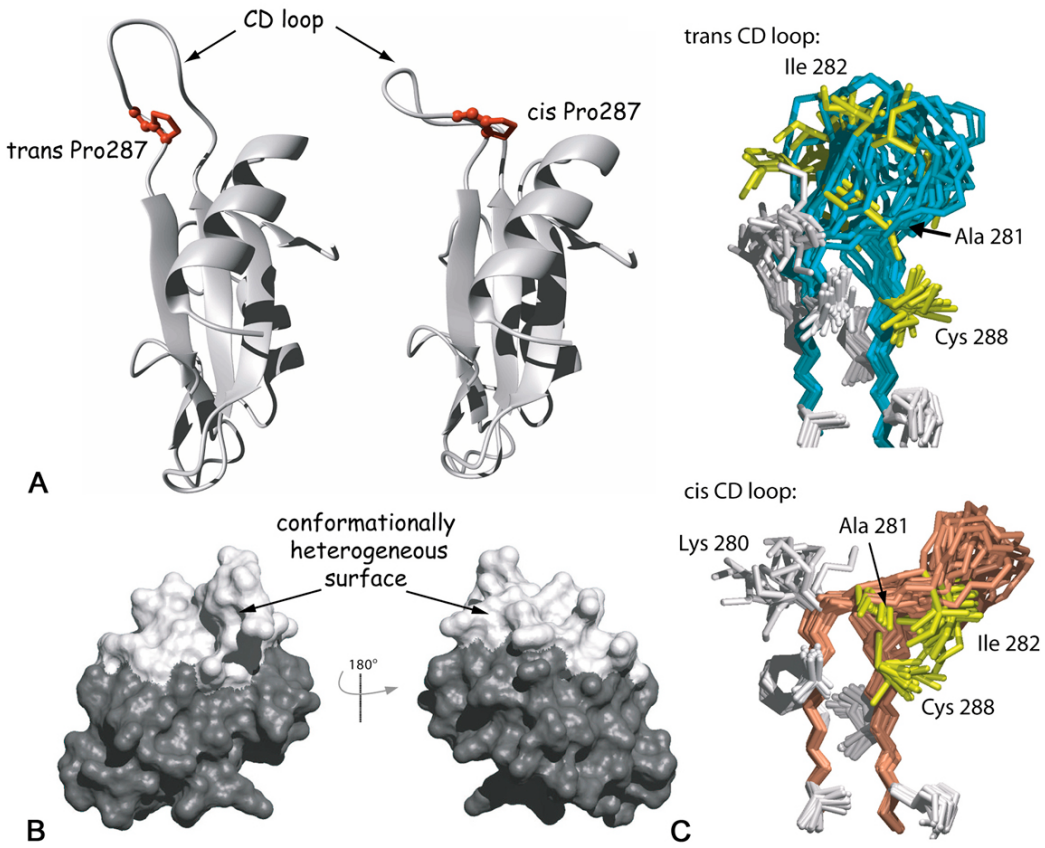

Figure 4. A. Ribbon representations of the energy minimized average structures of the *cis* (right) and *trans* (left) imide bond-containing SH2 conformers (pdb: 1LUK and 1LUN). The CD loop is labeled and Pro 287 is highlighted in ball and stick representation on each structure. B. Solvent-accessible surface plot of the cis conformer. The residues that give rise to dual resonances due to proline cis/trans isomerization are highlighted in white. The trans conformer shows a similar contiguous surface for the heterogeneous residues (not shown). C. Expanded views of the CD loop. Overlay of the energy minimized average NMR structures of the cis (coral) and trans (turquoise) conformers. Side chains of select CD loop residues are included and labeled. Ala 281, Ile 282 and Cys 288 are well defined and close in space in the cis form of the domain.

pocket that contacts the phosphotyrosine residue of the ligand and the 'pY+3' pocket that confers ligand-binding specificity by contacting amino acids flanking the phosphotyrosine (73, 74).

Unlike most SH2 domains, the function of the Itk SH2 module is not limited to canonical phospholigand binding. The Itk Src homology 3 (SH3) domain serves as an alternate ligand and binds to the Itk SH2 domain in an intermolecular, phosphotyrosine-independent manner (50). This interaction has been characterized for the single, separately expressed SH3 and SH2 domains (Figure 5a) as well as for larger fragments of Itk that include both domains. In the latter case, homodimerization of the Itk fragments is observed in solution (Figure 5b) and suggests that full length Itk likely self-associates via specific interactions between its regulatory domains.

Remarkably, dual ligand recognition by the Itk SH2 domain occurs in a conformer-specific manner (28, 60). Namely, the cis peptidyl-prolyl imide bond is preferred for the intermolecular interaction of the SH2 domain with the Itk SH3 domain and binding of a canonical phosphopeptide is mediated by the trans imide bond-containing SH2 domain. The conformer-specific nature of these recognition events is evident in NMR spectra of each protein/ligand complex. Addition of the Itk SH3 domain to the SH2 domain shifts the cis/trans ratio to favor the cis conformer, resulting in changes in the volumes of the NMR peaks corresponding to each conformer (Figure 5c). In contrast, binding of phosphopeptide to the Itk SH2 domain shifts the cis/trans equilibrium to favor the trans conformer. Thus, proline isomerization in the Itk SH2 domain controls the interaction of Itk with distinct ligands during cell signaling. To our knowledge, this is the first demonstration of *dual* ligand recognition that is governed exclusively by the conformation of a single prolyl imide bond within a folded protein receptor.

4. MECHANISTIC INSIGHTS INTO ITK REGULATION

4.1. Inactive Itk

We present here a model for Itk regulation that integrates much of the biochemical, kinetic and structural data obtained to date (Figure 6). At least two distinct

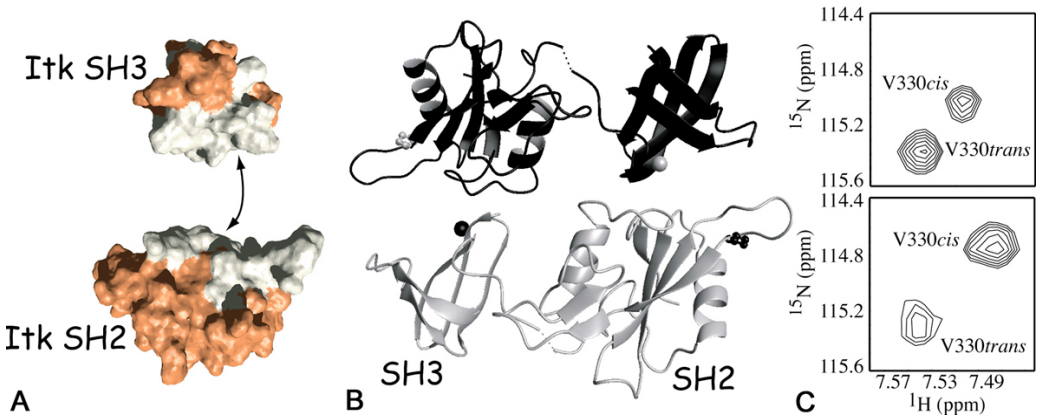

Figure 5. A. Solvent-accessible surface plots for the Itk SH2 and Itk SH3 domains where light shading indicates the residues involved in the intermolecular interaction as determined by chemical shift perturbations. B. A model for the Itk SH3-SH2 dimer based on chemical shift perturbations that define the dimer interface. (c) top panel, Select region of the two-dimensional HSQC spectrum of the free ^{15}N labeled SH2 domain. The region shown includes the doubled resonance for Val 330 where each peak is labeled according to whether it arises from the cis or trans forms. The peak volume reflects the population of each conformer in solution. Bottom panel, Same region of the HSQC spectrum of the ^{15}N labeled SH2 domain in the presence of excess Itk SH3 binding partner. SH3 binding shifts the cis/trans equilibrium to favor the cis isoform as measured by the change in relative peak volume.

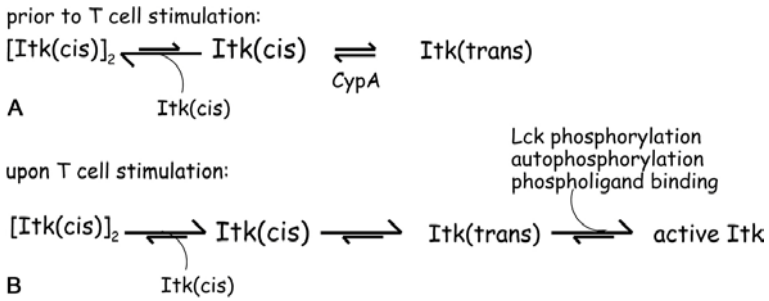

Figure 6. Model for Itk regulation. A. In the absence of activating signals the equilibrium between the cis and trans Itk conformers may shift toward the cis form by virtue of Itk self-association. $[Itk(cis)]_2$ refers to the self-associated form where intermolecular contacts between the regulatory domains include the cis SH2/SH3 interaction. Itk(cis) and Itk(trans) indicate monomeric protein that contains the cis and trans prolyl imide bonds, respectively. The model suggests that $[Itk(cis)]_2$ is the down regulated form of Itk. Cyclophilin A (CypA) may inhibit Itk by increasing the rate by which Itk(trans) is converted into Itk(cis) which is competent for self-association and down regulation. Alternatively, CypA stabilizes the inactive form of Itk through binding the inactive conformation. B. In response to TCR stimulation, Itk(trans) is captured by a transiently-phosphorylated signaling partner and phosphorylated both by Lck and by its own kinase domain (autophosphorylation) leading to upregulation of Itk catalytic activity. These activating interactions will shift the cis/trans equilibrium toward the trans conformer and away from the inactivating interactions mediated by the cis form.

species of the Itk kinase exist that differ with respect to the conformation about the imide bond preceding Pro 287. As described, the cis-SH2 domain binds specifically to the SH3 domain of another Itk molecule stabilizing homodimerization of the regulatory domains in solution (Figure 5). In this model, the self-associated species corresponds to inactive Itk kinase (Figure 6a). In analogy to the down-regulated form of the Src kinases (40), this conformation would mask the ligand binding sites of the SH3 and SH2 domains.

4.2. Itk activation by tyrosine 511 phosphorylation

Full activation of the Itk kinase following TCR stimulation is accompanied by phosphorylation at two specific tyrosine residues (75). One of these sites is Tyr 511, a residue that is located in the so-called activation loop of the Itk kinase domain and gets phosphorylated by Lck (76). In Itk-deficient primary T cells, a mutant that lacks this phosphorylation site (Itk Y511F) is completely unable to restore Itk mediated signaling (75).

The precise mechanism by which Y511 phosphorylation upregulates Itk remains unknown. Toward this goal, three-dimensional structures of the Itk kinase domain have recently been reported (77). The Y511 phosphorylated and unphosphorylated inhibitor-bound Itk kinase domains are structurally quite similar. Regardless of phosphorylation state, the Itk kinase domain appears to favor a conformation corresponding to that of an active kinase (77). This observation is not consistent with the severe defects observed in Itk Y511F transduced Itk$^{-/-}$ T cells that suggest that lack of phosphorylation at position

511 renders Itk completely unable to participate in T cell signaling. The Itk kinase domain structures also provide a strikingly different picture of regulation when compared to the Btk kinase for which phosphorylation-induced structural changes have been invoked to explain Btk regulation (78). Moreover, in the context of the Itk kinase domain structures, it is noteworthy that the isolated Itk kinase domain itself shows no activity in *in vitro* kinase assays (77, 79). It is therefore likely that the structures of the isolated Itk kinase domain (in the absence of the amino terminal regulatory domain sequence) do not provide a full understanding of phosphorylation-induced Itk regulation. A plausible conclusion is that phosphorylation of Y511 exerts its regulatory effect in concert with the non-catalytic regulatory domains of Itk and we must therefore await further biochemical analysis and structure determination of the full-length Itk protein to gain a full appreciation of the mechanistic details.

4.3. Itk activation by autophosphorylation at Y180 in the SH3 domain and phospholigand binding to the SH2 domain

In addition to phosphorylation at Y511, autophosphorylation at Y180 and binding of exogenous phospholigands accompany Itk activation following T cell stimulation (75, 80-82). Underscoring the importance of autophosphorylation, it has been shown that an Itk Y180F mutant can only partially restore Itk signaling in Itk-deficient primary T cells (75). Likewise, phospholigand binding to Itk (in particular binding of Itk to the linker of activated T cells (LAT)) is essential for Itk activation (81). Both of these activating events involve either the SH3 domain or the SH2 domain and both likely disfavor the self-associated form of Itk. Specifically, the autophosphorylation site, Y180, resides in the SH3 binding pocket – the same surface that mediates intermolecular binding to the cis SH2 domain. *In vitro* phosphorylation of the related Btk SH3 domain leads to significant changes in ligand binding affinities (83) suggesting a similar role for autophosphorylation in Itk. The Itk SH2 domain is required for Itk binding to LAT (a phosphoprotein) (81) and NMR data show that phospholigand binding to the Itk SH2 domain involves the trans form of the conformationally heterogeneous Pro 287 (60). During activation of Itk, engagement of the SH2 domain would sterically prevent the intermolecular interaction with SH3 while simultaneously destabilizing the cis form of the SH2 domain. Thus, via autophosphorylation in the SH3 domain and phospholigand binding to the SH2 domain, activated Itk is precluded from adopting a self-associated conformation characterized by the SH3 and SH2 mediated intermolecular interactions.

The essence of the proposed model is that the equilibrium characterizing the cis/trans prolyl isomerization is linked to two additional equilibria involving a distinct set of interactions for each of the conformers (Figure 6). In the absence of activating signals (i.e., Lck phosphorylation, autophosphorylation, LAT binding) the equilibrium will shift toward the inactive, cis conformer by virtue of high local concentrations of Itk that would favor self-association (Figure 6a). Isomerization to the trans form is likely to reduce Itk self-association affinity and could lead to one of two outcomes: (1) Itk could convert back to the cis conformer, re-associate and remain inactive (Figure 6a) *or* (2) if the appropriate signals emanate from the TCR, Itk would be poised for phosphorylation and LAT association leading to complete activation (Figure 6b).

4.4. Cyclophilin and dimerization of its target molecules : a putative mechanism for Itk regulation by CypA

In the context of the model we present here, the relatively high levels of Itk combined with the short-lived phosphoproteins that activate Itk following TCR stimulation (80) argue that the resting state of Itk is that of the self-associated form stabilized by the cis-Pro conformer. Thus, a putative role for CypA in this process is to increase the rate by which Itk is converted into the inactive, cis form (Figure 6a). CypA may promote Itk self-association by virtue of the linked equilibria controlling cis/trans isomerization and cis mediated dimerization.

This model for the effect of CypA in promoting Itk dimerization is analogous to the proposed mechanism for the control of channel activity of the cystic fibrosis transmembrane conductance regulator (CFTR) (84). In that case, channel activity is increased upon dimerization of CFTR. CFTR dimerization is favored by isomerization of three normally cis proline residues to the trans conformation. CypA appears to activate channel activity by promoting CFTR dimerization in much the same fashion as we propose here for CypA shuttling Itk toward the dimerized state.

The link between dimerization and peptidyl prolyl cis/trans isomerization has in fact been demonstrated for a number of systems. For example, dimerization of the C_H3 domain of the monoclonal antibody MAK33 requires the cis prolyl imide bond conformation at position 35 and association of the monomeric subunits to form dimer is accelerated by addition of a PPIase (85). Similarly, a proline switch has been shown to control domain interactions within the gene-3-protein of filamentous phage fd (86). In that case, as well as capsid assembly of the bacteriophage MS2 virus (87, 88), heterodimer formation within the Fv fragment of the phosphorylcholine binding antibody (89) and assembly of the cholera toxin B-subunit pentamer (90), it is the cis prolyl imide bond that is required for optimal association between domains. A number of proline residues occupy the dimer interface of the lambda Cro repressor and full assembly into dimers is dependent upon cis/trans isomerization (91). Even as early as 1981 investigators considered cis/trans isomerization of prolyl imide bonds as a rate limiting step controlling subunit association of mitochondrial malate dehydrogenase to form the dimeric functional enzyme (92). Finally, in addition to examples that directly implicate a specific prolyl imide bond conformation at an intermolecular interface, there are several examples in the literature that invoke a regulatory interaction between the cyclophilins and target proteins that either dimerize or oligomerize (93-98). Thus, it appears that cyclophilin may, in some cases, exert its regulatory role in conjunction with protein dimerization or oligomerization.

Given our current lack of mechanistic data for the Itk-CypA regulatory event, it should also be considered that CypA serves as a binding partner that sequesters Itk in its inactive conformation. Co-immunoprecipitation data from both Jurkat T cells and primary cells provide evidence for association between Itk and CypA (28, 58). The general question of whether CypA serves as a catalyst or binding partner has arisen for other systems (99) and to date remains largely unresolved. Further investigation of the mechanism of Itk regulation by CypA is certain to contribute to our general understanding of peptidyl-prolyl isomerase-mediated functions in cell signaling.

4.5. Why is Itk such an inefficient kinase *in vitro*?

Our model may also be used to shed light on the particularly sluggish nature of the Itk kinase (reported k_{cat} values range from 0.12-0.58 min^{-1} (77, 79)). A comparison with other tyrosine kinases (e.g., c-Src: k_{cat} is on the order of 20 sec^{-1} (100)) underscores the poor catalytic efficiency of full length Itk *in vitro*. While it is possible that the kinetic assays carried out to date made use of an inappropriate substrate, it is also conceivable that the experimental conditions of the *in vitro* kinase assay fail to mimic other physiological conditions that promote full activation of Itk.

For example, in the event that dimerization (or oligomerization) is a prerequisite for Itk activation, protein concentrations well below the dissociation constant for self-association will hamper the catalytic efficiency of the enzyme. Indeed, the reported kinetic assays for the Itk kinase have been carried out at low nM enzyme concentration (77, 79). Dimerization of an Itk fragment containing the SH3 and SH2 domains is characterized by a dissociation constant (K_d) in the μM range (50). While the oligomerization affinity for full length Itk is not known, it is quite likely that the K_d for this process is greater than low nM meaning that Itk is essentially monomeric under the conditions of the kinase assay. This model therefore invokes dual functionality for Itk dimerization. We have already suggested that the Itk dimer is the down-regulated form of the kinase in resting T cells and further suggest here that dimerization may be a necessary step toward Itk activation. For example, it is plausible that efficient Itk autophosphorylation will only occur under conditions that favor dimerization.

Alternatively, specific interactions mediated by the regulatory domains (for example SH2 domain binding to LAT) could serve to activate Itk by allosteric means. In the absence of appropriate ligands bound to the neighboring regulatory domains, the Itk kinase domain may only achieve partial activity leading to poor kinetic constants in vitro. Undoubtedly, the local cellular concentrations of Itk and required activators are precisely controlled during T cell signaling to either promote or disrupt Itk self-association as needed. Experimentally, in vitro conditions need to be identified that allow reasonable characterization of Itk enzyme kinetics in order to fully understand the intricate regulation of this kinase.

5. PERSPECTIVES

Proline is a chemically unique amino acid that has two thermodynamically accessible conformational states in the context of a native, folded protein. The Itk SH2 domain illustrates that 'native-state' proline isomerization can confer conformer-specific properties to a protein by modulating the features of a binding surface. The structural differences between the cis- and trans-containing conformers are exploited by incoming ligands and therefore confer dual functionality to a protein-binding surface at a reasonable energy cost. Dual protein functions arising from conformational plasticity in the absence of post-translational modification have been reported recently for unrelated signaling systems. One striking example is that of the Mad2 spindle checkpoint protein which adopts two distinct conformations in a proline-independent manner (101). Of particular interest with respect to Itk is the observation that the conformational change within Mad2 is also coupled to dimerization.

Future investigations of proline isomerization within the Itk SH2 domain as well as other conformationally heterogeneous proteins will undoubtedly shed light on the molecular basis for this intrinsic conformational exchange process. Are there primary, secondary and/or tertiary structural requirements for the occurrence of proline isomerization in the context of a folded protein such as Itk? How prevalent is this conformational switch among signaling proteins? Given that interconversion of prolyl conformers occurs spontaneously, what is the interplay between peptidyl prolyl isomerase activity and proline isomerization within folded proteins? Does functional regulation arise from the isomerase activity of CypA or does CypA fulfill its role in signaling by serving as a direct and stable binding partner?

Finally, in much the same way that mutation of tyrosine to phenylalanine disrupts important phosphorylation sites and abrogates signaling (102), it is possible that disease states will be identified in the future that arise from mutation of a functionally significant proline residue or even a proximal residue that stabilizes the cis population. Proline-dependent regulatory mechanisms would be susceptible to genetic alterations that render a polypeptide chain unable to access the cis conformation at a reasonable energy cost. Additionally, it should not be overlooked that the cis conformation of non-prolyl peptide bonds has been observed in several folded protein structures (103-108) and while isomerization of these peptide bonds does not appear to be catalyzed by the peptidyl prolyl isomerases (109), the unusual conformation occurs most often in functionally important sites (103). Thus, future efforts should strive to ascertain the extent to which native-state proline isomerization itself as well as population of the inherently less stable cis conformation of the imide or amide bond are general control mechanisms in cellular signaling.

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