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Analogous regulatory sites within the α C- β 4 loop regions of ZAP-70 tyrosine kinase and AGC kinases

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Summary

The precise positioning of the flexible C-helix in the catalytic core is a critical step in the activation of most protein kinases. Consequently, the α C- β 4 loop, which anchors the C-helix to the catalytic core, is highly conserved and mediates key structural interactions that serve as a hinge for C-helix movement. While these hinge interactions are conserved across diverse eukaryotic protein kinase structures, some families such as AGC kinases diverge from the canonical hinge interactions. This divergence was recently proposed to facilitate an alternative mode of regulation wherein a conserved C-terminal tail interacts with the α C- β 4 loop to position the C-helix. Here we show how interactions between the α C- β 4 loop and the N-terminal SH2 domain of ZAP-70 tyrosine kinase are mechanistically and functionally analogous to interactions between the α C- β 4 loop and the C-terminal tail of AGC kinases. Such *cis* regulation of protein kinase activity may be a feature of other eukaryotic protein kinase families as well.

Keywords

signaling; phosphorylation; catalysis; allostery; genome; evolution; inhibitors; dynamics; pathways

1. Introduction

Eukaryotic protein kinases (EPKs) control cellular signaling pathways through regulated catalytic transfer of a γ phosphate from ATP to the hydroxyl group of serine, threonine or tyrosine residues in protein substrates. Protein kinases are regulated through diverse mechanisms, each of which couples a specific upstream signal to that kinase's catalytic activity. For instance, the activity of protein kinase A (PKA) is regulated by a distinct regulatory subunit that interacts with the catalytic subunit in a cAMP dependent manner [1], whereas the activity of protein kinase B (PKB), which is closely related to PKA, is regulated by an N-terminal PH domain [2] that binds to phosphatidylinositol [3].

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Protein kinases can also regulate each other. For instance, activation of PKA and PKB involves phosphorylation by an upstream kinase called PDK1 (see review [4]), which docks to a C-terminal tail segment that is involved in positioning a key regulatory helix (the C-helix) within the catalytic core [5]. Displacement of the C-terminal tail from the C-helix of PKB causes kinase inactivation by disrupting a key salt bridge interaction between a glutamate that is generally conserved within the C-helix of protein kinases and a catalytic lysine, which coordinates with the phosphate group of ATP [6]. This mode of regulating protein kinase activity, by controlling C-helix movement, is also observed in other protein kinases such as Src tyrosine kinase, c-src tyrosine kinase (Csk) and cyclin dependent kinase 2. In Src tyrosine kinases and Csk, a linker region between the kinase domain and the N-terminal SH2 domain (which we term the SH2-kinase linker) repositions the C-helix [7]; whereas, in Cdk2 cyclinA repositions the C-helix [8].

Based on statistical analyses of protein kinase sequence divergence using the CHAIN program [9], we previously proposed structural features generally contributing both to the regulation of EPK activity [10] and to functional specificity of the CMGC [11] and AGC kinase families [12]. In particular, we noted that EPKs differ from distantly related eukaryotic-like kinases (ELK's) in that EPKs typically conserve a HxN motif within their α C- β 4 loop, which anchors the regulatory C-helix to the catalytic core. Based on available crystal structures, the HxN motif appears to mediate key structural interactions that serve as a hinge for C-helix movement. Although these hinge interactions are conserved across diverse protein kinase families, some families, such as the AGC kinases, diverge from the canonical interactions in the hinge region (and indeed lack the HxN motif). This divergent AGC structural feature was recently proposed to facilitate an alternative mode of regulation, wherein the conserved C-terminal tail interacts with the α C- β 4 loop and the C-helix to serve as a *cis* regulatory element. In this study, we propose that the ZAP-70 tyrosine kinase, which likewise lacks the HxN motif, is mechanistically related to AGC kinases inasmuch as ZAP-70 uses its N-terminal segment in a manner analogous to the C-terminal tail of AGC kinases. Our analysis suggests that such regulation of protein kinase activity by *cis* regulatory elements is typical of several other eukaryotic protein kinases as well.

2. The EPK HxN motif within the α C- β 4 loop

Several motifs within the catalytic domain are typically conserved within EPK's but not ELKs [10]. One of these is the HxN motif within the α C- β 4 loop (Fig 1A). In crystal structures, the HxN motif residues mediate key structural interactions that anchor the C-helix to the catalytic core. Specifically, the histidine (H60^{Cdk2}) within the HxN motif packs up against a conserved aromatic (F117^{Cdk2}) residue in the E-helix, while the HxN-asparagine (N62^{Cdk2}) hydrogen bonds to a conserved glutamine (Q110^{Cdk2}) in the E-helix. The HxN-asparagine also hydrogen bonds to the backbone carbonyl oxygen of a residue (I141^{Cdk2}) in the β 8 strand, which in turn forms a 'pseudo-beta sheet' that interacts [10] with the α C- β 4 loop (Fig 1B-C). Together, these lobe bridging interactions appear to anchor the flexible C-helix to the catalytic core.

2.1 The α C- β 4 loop as a hinge for C-helix movement

Comparison of the active with the inactive state of EPK's (Cdk2 in Fig 1B,C) indicates that the C-helix moves (as a rigid body), whereas the α C- β 4 loop region is relatively fixed. Moreover, distantly related ELK's, which lack the HxN motif, (such as aminoglycoside kinase (APH) in Fig 1D) exhibit a constitutively active C-helix conformation. Unlike EPKs, ELKs also harbor a conserved insert segment between the E-helix and the catalytic loop that interacts with the C-helix, apparently to stabilize it in an active conformation (Fig 1D). Thus the absence in ELK's of C-helix conformational flexibility and of the HxN motif suggests a possible role for the HXN motif in C-helix movement.

2.2 Integration of the C-helix hinge and inter-lobe hinge

The EPK α C- β 4 loop is also linked to a key salt bridge interaction between E81^{Cdk2} (Fig 1B-C) and K142^{Cdk2}, which based on an analysis of open and closed states of protein kinases structures was proposed to function as a hinge for inter-lobe movement [13]. Specifically, the backbone carbonyl oxygen of K65^{Cdk2} in the α C- β 4 loop hydrogen bonds to E81^{Cdk2}, and a conserved water molecule, which is surrounded by backbone atoms of the α C- β 4 loop, hydrogen bonds to K142^{Cdk2}. This structural link between the C-helix hinge (i.e., the α C- β 4 loop) and the inter-lobe hinge (i.e., the salt bridge between E81 and K142) could help coordinate inter-lobe movements (which typically occurs upon ATP binding) with C-helix movement (which occurs upon regulatory protein binding).

3. Sequence variation within the HxN motif reflect functional variation

A few EPK families, such as AGC kinases and ZAP-70 tyrosine kinases, lack the canonical HxN motif in the α C- β 4 loop region (Fig 2A, B). In the following sections we discuss these variations in light of existing structural data and propose that both AGC kinases and ZAP-70 tyrosine kinases employ an alternative mode of positioning the C-helix that involves the C- and N-terminal segments, respectively.

3.1 Regulation of AGC kinases by the C-terminal tail

PKA, PKB, PKC and closely related kinases form the AGC group within the eukaryotic protein kinase super-family [14]. A characteristic feature of AGC kinases is a conserved C-terminal tail [12] that wraps around the catalytic core to position key structural elements, including the C-helix (Fig 2C). Our previous comparison of AGC kinases with other eukaryotic protein kinases had revealed that nearly all the residues that distinguish AGC kinases from other eukaryotic protein kinases interact with the C-terminal tail [12]. This observation led to the hypothesis that the C-terminal tail (which we term the 'C-tail') has co-evolved with the catalytic core possibly to regulate AGC kinase activity. One of the residues that most distinguishes AGC kinases from other eukaryotic protein kinases is a conserved phenylalanine (F102^{PKA}), which replaces the HxN-asparagine in the α C- β 4 loop (Fig 2A). This variation appears to facilitate an alternative structural arrangement in which the C-terminal tail directly interacts with the α C- β 4 loop (Fig 2C). Specifically, the aromatic ring of F102^{PKA} in the α C- β 4 loop maximally packs up against a conserved basic residue (R308^{PKA}) in the C-terminal tail, thereby allowing the basic residue to directly hydrogen bond to the backbone carbonyl oxygen of P101^{PKA} in the α C- β 4 loop. Why would such an interaction be essential for AGC kinase functions? One possibility is that these interactions (between the α C- β 4 loop and the C-tail) may facilitate coordination of C-helix with the C-tail movement as both these structural element (C-helix and the C-tail) undergo dramatic conformational changes during the activation process of PKB [6]. Although these observations are suggestive, a complete understanding of AGC specific variations in the α C- β 4 loop will require a combination of mutational and solution studies.

3.2 Regulation of ZAP-70 and Syk tyrosine kinases by the N-terminal SH2 domain

The cytoplasmic tyrosine kinase ZAP-70 is involved in T-cell antigen receptor signaling [15]. A characteristic feature of ZAP-70 and of the related spleen tyrosine kinase (Syk) is a tandem SH2 domain arrangement that is located at the N-terminus of the catalytic domain. This domain arrangement is important for ZAP-70 and Syk functions as the N-terminal SH2 domains directly interacts with the catalytic domain to regulate activity [16]. CHAIN analysis reveals that some of the residues distinguishing ZAP-70 and Syk from other tyrosine kinases are located in the α C- β 4 loop (Fig 2B). In particular, the canonical HxN motif in the α C- β 4 loop is conserved as a NxY motif in ZAP-70 and Syk kinases. This sequence variation appears to reflect the functional variation of ZAP-70 inasmuch as the NxY motif directly interacts with the SH2-kinase linker, which contains a phosphorylatable tyrosine (F/Y319^{ZAP-70} in Fig 4D),

mutation of which prevents ZAP-70-dependent T cell signaling [17]. In particular, F/Y319^{ZAP70} comes in close proximity to the NxY-tyrosine in the crystal structure and packs up against a proline, which is located at the “x” position within the NxY motif (not shown). These and other interactions between the α C- β 4 loop and the SH2 domain were proposed to stabilize the C-helix of ZAP-70 in an inactive conformation [16]. This mode of positioning the C-helix is strikingly similar to AGC kinases wherein the C-tail interacts with the N and C-terminal either ends of the C-helix to position it in an active conformation (Fig 2C).

3.3 Regulation of MEK1 and ErbB2 may also involve cis-regulatory elements

In addition to AGC kinases and ZAP-70, several other eukaryotic protein kinase families also lack the canonical HxN motif in the α C- β 4 loop. Some of these include the Map kinase kinase 1 (MEK1s) and the ErbB2 receptor tyrosine kinases. MEK1 conserves a SxY motif instead of the HxN motif, while ErbB2 conserves a CxY motif at the corresponding position. Notably, both MEK1 and ErbB2 contain flanking segments that are known to play regulatory roles [18,19]. Thus, the regulation of MEK1 and ErbB2 seems likely to involve interactions between the α C- β 4 loop and the flanking regions. Future studies will focus on how these flanking segments have co-evolved with the catalytic core to regulate activity.

4. Experimental Approaches

CHAIN analysis of EPK's, AGC kinases and ZAP70 tyrosine kinases

Evolutionary constraints imposed within the α C- β 4 loop region of EPKs (Fig 1A), AGC kinases (Fig 2A) and ZAP-70 tyrosine kinases (Fig 2B) were measured using a Bayesian statistical approach called CHAIN analysis [9]. The results are displayed using a Contrast Hierarchical Alignment, which is based on three categories of related sequences: (i) a foreground set (ii) a display set and (iii) a background set. Category specific constraints are measured by the degree to which residues in the foreground set contrast with residues observed at the corresponding position in the background set. Therefore, foreground positions with compositions that closely resemble the background have little or no category-specific constraints while compositions that strikingly “contrast” with the background are subject to strong category-specific constraints (as indicated by the height of the histogram above the alignment in Fig 1A and 2A-B). For reviews of CHAIN analysis and of the CHAIN program see [9].

5. Conclusions and perspectives

A comparative analysis of protein kinase families has revealed a regulatory role for the α C- β 4 loop in EPK functions. The α C- β 4 loop appears to not only facilitate C-helix movement but also integrate C-helix movement with regions that serve as hinges for inter-lobe and activation loop movement. Not surprisingly, segments such as SH2 domain in ZAP-70 tyrosine kinase and the C-tail in AGC kinases interact with the α C- β 4 loop apparently as a mode of regulation. These mechanistic differences between EPK's, ELK's and some EPK families are reflected at the sequence level and can be detected statistically. Finally, the proposed regulatory role of the α C- β 4 loop opens up new avenues of exploration for the design of allosteric inhibitors.

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Abbreviations

AGC kinases

PKA, PKG, PKC and related kinases

ZAP-70 tyrosine kinase

Zeta-chain-associated protein kinase 70

Syk

Spleen tyrosine kinase

MEK1

Map kinase kinase-1

ErbB2

erythroblastic leukemia viral oncogene homolog 2

SH2

Src homology domain 2

PH

Pleckstrin homology domain

Cdk2

cyclin-dependent kinase 2

CMGC

Cyclin-dependent kinases, Mitogen activated protein kinase, Glycogen synthase kinase-2 and Ck2

EPK

Eukaryotic protein kinase

ELK

Eukaryotic-like kinases

Figure 1.

Selective conservation of the HxN motif in the α C- β 4 loop region and its structural location in Cdk2 (EPK) and aminoglycoside kinase (ELK). **A.** A contrast hierarchical alignment showing evolutionary constraints imposed within the C-helix and the α C- β 4 region of EPK's. All EPK sequences (14,667) constitute the foreground set in the alignment, while all ELK sequences constitute the background set. Representative sequences from various EPK families are used as the display set. The histogram above the alignment measures the degree to which aligned residue positions in the foreground set are shifted away from the corresponding position in the background set. Residue positions subject to the strongest constraints are highlighted with chemically similar conserved amino acid residues colored similarly; very weakly

conserved positions and nonconserved positions are shown in dark and light grey, respectively. Dots below the histograms indicate those residues positions that most strikingly distinguish EPKs from ELKs, as selected by our statistical procedure[9]. The foreground set includes both EPK sequences shown in the alignment and other EPK sequences, the numbers of which (after adjusting for sequence redundancy) are denoted by their weighted residue frequencies ('wt_res_freqs') below the alignment. Residue frequencies are indicated in integer tenths where, for example, a '7' indicates that the corresponding residue directly above it occurs in 70-80% of the (weighted) sequences. Similarly, the background set (ELKs) is shown below the foreground alignment. NCBI sequence identifiers used in alignment 1A are: 6730497|Cdk2-human; 20150484|Cdk6-human; 1110512|Erk2-rat; 24987248|Gsk3b-human; 6137569|FGF-human; 15988251|Tie2-human; 30749934|C-Abl-mouse; 3114436|CalMK-rat; 46397802|Aurora1K-yeast; 38016021|STK-plant; 47169341|B-Raf-human; 15988011|TGF-human. **B.** Structural interactions that anchor the α C- β 4 loop to the C-lobe in the active state of Cdk2 (PDB: 1QMZ). **C.** Structural interactions in the α C- β 4 region of inactive Cdk2 (PDB: 1HCL). **E.** The α C- β 4 loop associated interactions in aminoglycoside kinase (PDB: 1J7L). The structural figures shown in Fig 1(B-E) and Fig 2(C-D) were generated using Pymol [20]. Hydrogen bonds are indicated by dotted lines and residues are colored using a coloring scheme as indicated in the alignments. The EPK-ELK shared residues are shown in magenta. EPK-specific residues are shown in gold. Group specific residues are shown in yellow, while family specific residues are shown in cyan. The C-helix is denoted as α C.

Figure 2.

Sequence variation within the α C- β 4 loop region reflects functional variation in AGC kinases and ZAP-70 tyrosine kinase **A**. Contrast hierarchical alignment showing evolutionary constraints imposed within the α C- β 4 region of AGC kinases. These constraints were measured using all AGC kinase sequences as the foreground set and all eukaryotic protein kinase sequences as the background set. Representative sequences from various AGC kinase families constitute the display set. NCBI sequence identifiers used in alignment 2A are: 125205|PKA_human; 1170703|PKB_human; 20141582|PKCT_human; 417080|GRK_fruitfly; 47605999|RHOK_human; 7649389|RSK_plant; 56749457|NDRK-human **B**. Evolutionary constraints imposed within the α C- β 4 region of ZAP70 kinases and related Syk kinases. In this

alignment, the foreground set corresponds to ZAP-70 and Syk tyrosine kinase sequences, while the background corresponds to all tyrosine kinases. Representative sequences from different phyla of ZAP-70 and Syk constitute the display set. NCBI sequence identifiers used in alignment 2B are: 1177044|ZAP70-human; 26453338|ZAP70-mouse; 50416266|ZAP70-frog; 50761018|ZAP70-chicken; 47086347|Syk-zebrafish; 50761846|Syk-chicken **C.** Backbone and side-chain interactions in the α C- β 4 region of PKA showing the integration of the C-terminal tail with the hinge region. The hydrophobic motif (HF-motif) in the C-tail and its docking interaction with the C-helix is also shown (PDB: 1ATP). **D.** Specific interactions between the SH2 domain and the α C- β 4 region of ZAP70 tyrosine kinase (PDB: 2OZO). Residue coloring are as indicated in Fig 1 caption.