Structural basis of regulation and substrate specificity of protein kinase CK2 deduced from the modeling of protein-protein interactions

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Abstract

Background: Protein Kinase Casein Kinase 2 (PKCK2) is an ubiquitous Ser/Thr kinase expressed in all eukaryotes. It phosphorylates a number of proteins involved in various cellular processes. PKCK2 holoenzyme is catalytically active tetramer, composed of two homologous or identical and constitutively active catalytic (α) and two identical regulatory (β) subunits. The tetramer cannot phosphorylate some substrates that can be phosphorylated by PKCK2α in isolation. The present work explores the structural basis of this feature using computational analysis and modeling.

Results: We have initially built a model of PKCK2α bound to a substrate peptide with a conformation identical to that of the substrates in the available crystal structures of other kinases complexed with the substrates/pseudosubstrates. In this model however, the fourth acidic residue in the consensus pattern of the substrate, S/T-X-X-D/E where S/T is the phosphorylation site, did not result in interaction with the active form of PKCK2α and is highly solvent exposed. Interaction of the acidic residue is observed if the substrate peptide adopts conformations as seen in β turn, α helix, or 3_10 helices. This type of conformation is observed and accommodated well by PKCK2α in calmodulin where the phosphorylation site is at the central helix. PP2A carries sequence patterns for PKCK2α phosphorylation. While the possibility of PP2A being phosphorylated by PKCK2 has been raised in the literature we use the model of PP2A to generate a model of PP2A-PKCK2α complex. PKCK2β undergoes phosphorylation by holoenzyme at the N-terminal region, and is accommodated very well in the limited space available at the substrate-binding site of the holoenzyme while the space is insufficient to accommodate the binding of PP2A or calmodulin in the holoenzyme.

Conclusion: Charge and shape complementarity seems to play a role in substrate recognition and binding to PKCK2α, along with the consensus pattern. The detailed conformation of the substrate peptide binding to PKCK2 differs from the conformation of the substrate/pseudo substrate peptide that is bound to other kinases in the crystal structures reported. The ability of holoenzyme to phosphorylate substrate proteins seems to depend on the accessibility of the P-site in limited space available in holoenzyme.
**Background**

Protein Kinase Casein Kinase 2 (PKCK2) is a ubiquitous Ser/Thr kinase expressed in all eukaryotic cells. It can phosphorylate a large number of substrates localized either in nucleus or cytoplasm. The substrates of PKCK2 are known to be involved in various cellular processes like signaling, transcription and translation control, structural proteins and oncogene response in the cell [1,2]. The enzyme is highly conserved in evolution, and the lowest pairwise sequence identity between two members of PKCK2 sub-family is as high as 40%. The catalytic subunit of PKCK2 shows high sequence similarity to CDK subfamily of kinases although the nature of regulation is entirely different between PKCK2 subfamily and CDK subfamily [2]. PKCK2 knockout has been shown to be lethal in baker’s yeast [3] and recent micro-array experiments have shown that PKCK2 is interacting with transcription control elements [4].

PKCK2 consists of two types of subunits: the catalytic α subunit and the regulatory β subunit [1,2]. The β subunit can form homodimer which can associate with two α subunits and the tetramer of two α and two β subunits form the holoenzyme [5]. The formation of holoenzyme is spontaneous within cells [6,7]. Holoenzyme is also catalytically active, but holoenzyme alone cannot phosphorylate some of the substrates like calmodulin. The phosphorylation of these substrates however, is carried out in the presence of polycations like polylysine [7]. Some of the positively charged compounds, like spermine, that has been reported to stimulate the PKCK2 mediated phosphorylation of a transcription factor MyoD in vitro [8], results in major structural changes in the PKCK2 [9].

The signature pattern of the substrate that is recognized by the catalytic subunit of PKCK2 is S/T-X-X-E/D where the Ser or Thr is the residue that is phosphorylated and X represent non-basic residues [1,2]. However Tyr phosphorylation by PKCK2 has also been reported [10]. It is also known that the acidic residue at the fourth position in the signature pattern can be replaced by phosphorylated Ser/Thr/Tyr [2,11]. This indicates that PKCK2 can participate in hierarchical phosphorylation of substrates by other kinases [11].

PP2A2 is a protein phosphatase that is involved in the down regulation of MAPK cascade by de-phosphorylating the MAPK and MEK or the MAPKK. The core PP2A is a heterodimer of catalytic PP2Ac subunit and an invariant regulatory subunit referred to as PR65. Another regulatory subunit, PP2B, binds to PR65 and is thought to confer substrate specificity to the PP2Ac [12]. It has been suggested that PP2Ac binds to PKCK2α and not the holoenzyme [13,14]. The possibility of PP2Ac being a substrate to PKCK2α has been raised [13–15]. In the present work we suggest structure-based reasons for the binding of PP2Ac more favourably to PKCK2α than to holoenzyme. We provide structure-based reasoning also for the capability of PKCK2α binding and phosphorylation of calmodulin and for the inability of the holoenzyme to bind and phosphorylate calmodulin.

Structural modeling and analysis of PKCK2 have provided useful information in the past [16,17]. In the current work we present the structural models of interactions between PKCK2α and some of its substrates or binding proteins. Our aim is to generate models of PKCK2-substrates/binding protein complex in order to understand the modes of binding and protein-protein interactions involving PKCK2. To this end, we surveyed the database of known structures for the availability of 3-D structures for PKCK2 binding proteins. If the binding protein is a substrate then the knowledge of the phosphorylation site is also required. Thus, we arrive at three cases, calmodulin, PKCK2β and PP2Ac all of whose structures or structure of close homologues are available. The conformation of peptide bound to PKCK2α is currently unknown and hence we have first generated and analyzed number of conformations of the peptide with the characteristic S/T-X-X-D/E sequence bound to PKCK2α. Based on the models of PKCK2α bound to substrate peptides and the shape and charge complementarity between the substrate-binding site and the substrate proteins, we have generated and analyzed models for PKCK2α bound to calmodulin, and PP2A. We have also modeled the interaction between PKCK2α and PKCK2β when the latter gets phosphorylated during the autophosphorylation of PKCK2.

**Results and Discussion**

**Modeling of PKCK2 bound to substrates in different conformations**

As mentioned earlier the essential peptide sequence pattern for the phosphorylation by PKCK2 is S*/T*/X-X-D/E where S/T is the phosphorylation site or P-site. The P+3 site should be acidic and P+1 and P+2 site should be non-basic. In the available crystal structures of other protein kinases, such as cyclic AMP-dependent protein kinase (cAPK), cyclin dependent kinase (CDK) and phosphorylase kinase complexed with substrates or pseudo substrates, we find that the residues at the P+1, P+2 and P+3 sites all have comparable main chain torsion angle (ϕ, ψ) values and falls in the extended region of the Ramachandran map. We have docked the substrate peptide of PKCK2 in the same conformation and mode as that observed in the complex crystal structures of other kinases, in the crystal structure of PKCK2α. (See the section on Methods for the details). As PKCK2α is constitutively active the available structures of PKCK2α also corresponded to the active conformation of the enzyme. In the modeled structure of the
complex, with the substrate peptide in the same conformation and position as in cAPK and phosphorylase kinase, favorable interactions are present between the residues at P+1 and P+2 site and the residues of PKCK2α. However the critical acidic residue at P+3 site is not interacting with any of the residues in the kinase and is solvent exposed. However, the site P+3 shows good interaction with the 74–77 basic cluster conserved in the PKCK2 sub-family, if the peptide adopts the same mode of binding as seen in CDK complexed with its substrate peptide as reported earlier [18,19]. The acidic residue at the position P+3 is well conserved within almost all known PKCK2 substrates. Hence it could be assumed that this residue interact with a basic residue in the PKCK2α that is conserved within the PKCK2α sub-family. Analysis of the known substrates (data not shown) of PKCK2 shows that at position P+3, D is preferred but next only to E. Only in two cases we find that this site is occupied by A or G, both non-acidic amino acids (HMG14 and in clathrin light chain LCβ), and experimental data shows that the phosphorylation of HMG14 by PKCK2 is sluggish [20]. As dealt later in this paper the known 3-D structure of the substrate calmodu-

Table 1: The secondary structure prediction in the known PKCK2 phosphorylation sites (S/T-X-X-D/E) in protein substrates. H represents prediction of helix and – represent no prediction of helix or β-sheet. Prediction by both PHD and JPRED methods have been shown in those cases with difference in the secondary structure prediction.

| Protein          | Secondary structure prediction at the S/T-X-X-D/E motif |
|------------------|----------------------------------------------------------|
| Acetyl Co-A      | ---HH                                                    |
| Clathrin         | 11,13 -HH                                                |
| EF               | 90 -HH                                                   |
| Hsp90 (beta)     | 226, 255 --HH                                            |
| Hsp90 (alpha)    | 236,263 --H, ---H                                       |
| EIF              | ---                                                      |
| CK2β             | ---                                                      |
| DRAPP32          | 42,45,102 ----, ----                                    |
| HPr E7           | 31,32 ----                                               |
| Troponin T       | 1 ----                                                   |
| Glycogen synthase| 657 ----                                                 |
| PKA RII          | 74,76 ----                                               |
| HMG14            | 89 ----                                                  |
| Myosin light chain| --HH (PHD) -HHH(JPRED)                                  |
| ODC              | 297,303 ----                                             |
| Ppi              | 87,120,121 ----                                         |
| Calmodulin       | 81/79,101 HHHH, ---HH                                    |
| MDM2             | 267 ----                                                 |
| PS3              | 392 ----                                                 |
| Eif 2 beta       | 2.67 ----                                                |
| CB subunit of MCP| 243, 250 HHHH, ---HH                                    |
| elf5             | 387, 388 ----                                           |
| FCP1             | 457 ----                                                 |
| Movement protein of TMV | 256,261 ----, ----                                   |
| CDC25B           | 186,187 ----                                             |
| FMR1             | 406 ----                                                 |
| PTEN             | 370,385 ----                                             |
| Mengovirus leader protein | 47 ----                         |
| β arrestin 2     | 383 --HH (JPRED) ----(PHD)                               |
| Cyclin H         | 315 ----                                                 |
| UBC3B            | 233 ----                                                 |
| Topoisomerase II α | 1342 ----                                      |
| Rotavirus NSP5   | 153,155,163,165 ---, ---, ---, ---                       |
| TF III A         | 16 ----                                                  |
| HIV1 Rev         | 5,8 ---- (JPRED) HHHH (PHD), -HHH (JPRED) HHHH(PHD)     |
| HDAC1            | 421,423 ----(JPRED) ---H (PHD), ----(JPRED) HHHH (PHD) |
| CDC34            | 203,222,231,233,236 ---, ---, ----, ----, ----          |
| elfR epsilon     | 712,713 ----                                              |
| FAF1             | 289,291 ----                                             |
| VIPR-RP          | 69,71,110 ----                                           |
| NDPKA            | 122 ---                                                  |

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lin and secondary structure prediction of a number of substrates raise the possibility of PKCK2α phosphorylation site being in a helical or, in more general terms, turning region. It is perceivable also from the strong restriction imposed at the P and P+3 sites of the consensus substrate sequence that these positions are facing towards the catalytic site with the middle two residues somewhat exposed with no essential requirement to interact with the enzyme. Thus, the attractive conformations that would orient the P and P+3 residues in approximately the same direction as the substrate-binding site is the turning conformations as in β-turn and α or 3_10 helices. The secondary structure prediction of the 41 substrate sequences, with known PKCK2α phosphorylation sites, shows that 50 sites out of 67 sites in 41 substrate proteins are involved in connectors of helices and extended-strands and so they make turns and loops. Five sites (of the 67 sites) lies completely within helices and another 12 sites are at the beginning of helices (Table 1). The secondary structure prediction results however do not discriminate among β-turns, loops and extended strands not involved in β-sheet formation. Although type I/III β-turn conformation can be perceived as a very short segment of a "helix" such turns are usually predicted as a "loop" by most secondary structure prediction programs.

To explore the possibility of PKCK2α substrates adopting a turning conformation, model β-turn peptides (type II, III and I) have been generated using PEPGEN (C. Ramanarikrishnan, unpublished). Each of these peptides in turning conformation has been placed in the catalytic PKCK2α [24] in the same orientation of Ser residue as that of the Ser 4 of substrate peptide-CDK complex whose crystal structure is already available [25]. In the modelled complexes the direction of peptide is maintained as in crystal structure complexes. In all the cases, the P+3 acidic residue is in good contact with the basic residues Arg 191 and Arg 195 of the kinase and one of the modelled complex structures is shown in Figure 1. Arg 191 and Arg 195 are almost completely conserved within the sub-family of PKCK2α (data not shown).

The β-turns are generally found in proteins where chain reversals take place. In the cases of larger substrates such as proteins binding at the PKCK2α active site, a chain reversal without further turning conformations is not favourable at the substrate-binding site with phosphorylatable serine as the first residue of the turn. This is because, though it is essential to have the four-residue signature for effective binding to PKCK2α, it is also noted that effective optimum binding is achieved with longer peptides [2]. Thus it is suggested that, in the case of large substrates as proteins, a turn alone cannot be accommodated well at the substrate-binding site and the downstream elements also bind to the enzyme. If the residues at the C-terminal of the phosphorylatable Ser/Thr adopts the same or similar conformation as that of the four residue signature sequence, such that the gross direction of the backbone is maintained, then effective binding at the substrate binding cleft is achieved.

Substrate analysis

From the crystal structure of the holoenzyme [26] it is clear that the access to the active site is possible only if the phosphorylatable segment is adopting a conformation to enter the catalytic site as well as if the substrate binding is not interfered by the neighbouring β-subunit in the tetramer. A sufficiently long looping segment is an attractive possibility as it could protrude into the catalytic site of the enzyme. Entry of a helix or a region involved in the β-sheet in the middle of the structure of a substrate protein into the catalytic site would be difficult as rest of the substrate might have short contact with the β-subunit. Further, if the phosphorylatable residue is located close to the termini of the polypeptide chain the access to the catalytic site might become easier and the chances of short contact with the β-subunit is low. To explore such possibilities, a
survey of known PKCK2 substrates, whose PKCK2 phosphorylation sites are well documented, and the enzyme system (holoenzyme or PKCK2α alone) catalyzing the phospho-transfer reaction is clearly understood, has been made. We had surveyed the lists of Allende and Allende [1], Pearson and Kemp [27] and various other literature reports for the substrates, which satisfy the requirement of knowledge of phosphorylation site and enzyme system. As most of the PKCK2 substrates did not have a known three-dimensional structure secondary structure prediction has been carried out using the PHD [21,22] and JPRED [23] to understand the preference of substrate conformation. The state of the enzyme that carries out the phosphorylation was also obtained from the literature as referenced in Table 2.

From the Table 2, it is clear that holoenzyme catalysis generally occurs at the termini and in predicted loop regions. Although the α-subunit alone could also phosphorylate these substrates, the Ser/Thr of many substrates that are phosphorylated by α-subunit (without the aid of β-subunit) are at the central region of the polypeptide chain away from either of the amino or carboxy terminus and predicted to adopt helical conformation. It is perceivable that the helical conformation and the location of Ser/Thr in the middle of a polypeptide chain render the access of this region difficult for the catalytic sites in the holoenzyme. Hence it is predicted that the variation in specificity of PKCK2α and holoenzyme towards various substrates is due to restricted access to the active site. However, if major gross structural changes (either in substrate and/or holoenzyme) are brought about, the phosphorylation can be carried out by holoenzyme in the first cases as well.

**PKCK2α binding to calmodulin**

Calmodulin undergoes a number of post-translational modifications such as acetylation, methylation, carboxy methylation and proteolysis. Plancke and Lazarides [28] identified phospho-calmodulin in chicken brain cells. They also found that calmodulin is also phosphorylated in vitro by phosphorylase kinase purified from skeletal muscle cells. Phosphorylation of calmodulin by PKCK2 in vitro was shown by Nakajo et al [29] and by Meggio et. al [30]. Marin et. al [31] showed that the phosphorylation site of PKCK2 on calmodulin is Ser 81 by detailed analysis of phosphorylation of calmodulin fragments by PKCK2α alone as well as with holoenzyme. Recently, it has been observed that calmodulin is phosphorylated in vivo at the same site [32] and this affects the activation of certain calmodulin dependent enzymes [33].

In the present work, we have built models of PKCK2α bound to calmodulin and explain the structural basis of phosphorylation. The methodologies adopted to obtain models are described in *Methods* section. In the models built, as shown in figure 2, we see that the central helix of calmodulin fits very well into the substrate-binding site of PKCK2α. All the PKCK2 basic residues reported [18,19] to be interacting with substrate are in good interacting distances. Ser 81 is in the interacting distance with catalytic base of PKCK2α, Asp 156. Other basic residues of PKCK2α reported, Arg 191, Arg 195, Lys 198 are interacting with Glu 82 and Glu 84 of calmodulin. Glu 82 from calmodulin also interacts with Lys 49, another important substrate binding residue of PKCK2α. The other potentially interacting residues from calmodulin is Glu 139, which is in close proximity of Lys 74 of PKCK2α. Asp 156, Glu 54 and Glu 87 of calmodulin are spatially close to Arg 43 of PKCK2α. There are no hydrophobic patches exposed in the complex structure or no like charges in close proximity are noticed in the potentially interacting regions. The mode of binding here seems to be driven by the charge and shape complementarity between the enzyme and the substrate. As seen from figure 3a and figure 3b, the negative charges are distributed all over the surface of calmodulin and PKCK2α is positively charged on its surface.

The phosphorylation of calmodulin by PKCK2α is down-regulated by the PKCKβ subunit. It is observed in the models of PKCK2 holoenzyme bound to calmodulin

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| Substrate   | Phosphorylation sites | Phosphorylating system (PKCK2α or holoenzyme) | PHD Result | How far from C/N termini | Ref No. |
|-------------|----------------------|----------------------------------------------|------------|--------------------------|--------|
| Calmodulin  | S81, T79             | α                                            | Helix      | Central region           | 7,27   |
| NDPKA 151 aa| S122                 | α                                            | Helix      | ~30aa away from the C-terminal | 61 |
| NAPI DROM 370 aa | S284             | α                                            | Loop       | Central region           | 62 |
| C8 of MCP 255 aa | S250            | Holoenzyme                                   | Loop       | C-Terminal region       | 63 |
| BTF3a 206 aa  | T182                | Holoenzyme                                   | Loop       | C-Terminal region (20 aa away) | 64 |
| HIV I Rev 116aa | S5, S8         | Holoenzyme                                   | Helix      | N-Terminal region       | 41, 42, 43 |
| PKCK2ji     | S2                  | Holoenzyme                                   | Loop       | N-Terminal region       | 36 |
that the presence of PKCK2β subunit causes steric hindrance with the calmodulin bound to PKCK2α, especially in its C-terminal region. There is a high population of negatively charged amino acids on the surfaces of calmodulin and PKCK2β. Thus, the surface charges might also repel the two proteins from coming in close contact. Thus, in presence of PKCK2β, the calmodulin might be displaced from the PKCK2α binding site (as a result of high binding affinity of PKCK2β to PKCK2α [6,7]), which explains the down regulation observed. However, in the presence of polybasic compounds that binds effectively to the PKCK2α subunit [34,35], the surface negative charges on PKCK2β might be masked. If this interaction is also coupled with some structural variation in the holoenzyme, such that calmodulin binding is well tolerated by the holoenzyme, then phosphorylation of calmodulin by holoenzyme is possible. This might explain the experimental observation that calmodulin phosphorylation happens in the presence of polybasic compounds by the PKCK2 holoenzyme [7].

Auto-phosphorylation
PKCK2 holoenzyme is known to phosphorylate the PKCK2β subunit [1,36]. This auto-phosphorylation down regulates the PKCK2 activity. The phosphorylation site in the β-subunit is at the N-terminal (Ser 2). This site is visible in the electron density corresponding to one of the β-chains in the crystal structure of holoenzyme [26]. In our model Ser 2 has been placed at a hydrogen bonding orientation from the catalytic base Asp 156 of the α-subunit. Further, the downstream acidic charges are interacting with basic substrate binding determinants. The model of PKCK2α binding to PKCK2β in substrate binding position is generated as discussed in Methods and is shown in figure 4. The charges on the surface of the β-subunit compliments with the surface charges of α-subunit, with positive charges on α-subunit and negative charges on the β-subunit as shown in figure 5.

The mode of phosphorylation of PKCKβ by the holoenzyme in terms of intra tetramer (cis) nature or inter tetramers (trans) nature is not clear from literature. Considering intra-tetramer (cis) mode of phosphorylation, in the available crystal structure of holoenzyme [26], the distance between the phospho-acceptor site on PKCK2β and the catalytic base on PKCK2α is about 50.5 Å, a distance that appears cannot be reduced even by reasonably large conformational changes in the holoenzyme to bring the PKCK2β phospho-acceptor site close to active site. Thus, within a tetramer of PKCK2 the phosphorylation site in the β-subunit is far away from the catalytic base in the α-subunit and hence the cis phosphorylation appears unlikely. Hence, based on the available crystal structural data on holoenzyme, we assume that the PKCK2β phosphorylation by the holoenzyme on Ser 2 could be inter-tetramers (trans).
From the modeling studies it is suggested that the phosphorylation of $\beta$-subunit is 'trans auto' with one molecule of holoenzyme phosphorylating the $\beta$ subunit of another holoenzyme molecule forming a transient dimer of tetramers. A rough model of PKCK2 holoenzyme with PKCK2$\beta$ bound to it as a substrate has been generated and is as shown in figure 6. From the figure, it can be seen that the PKCK2$\beta$ from a neighbouring holoenzyme molecule can be comfortably accommodated at the substrate-binding site. Location of Ser 2 in the $\beta$-subunit is exposed in such a way that it does not prevent the holoenzyme to phosphorylate it.

In the 3-D models of holoenzyme bound as substrate (R – Receiver of phosphate) to another copy of the holoenzyme (D – Donor of phosphate) that is generated as in figure 6, we find that the positioning of the substrate holoenzyme (copy R) is in such a manner that it could phosphorylate the PKCK2$\beta$ of the other copy of the holoenzyme (copy D). The model generated turns out to be a symmetric complex with two copies of holoenzyme that can potentially phosphorylate each other.

The autophosphorylation is known to reduce the activity of PKCK2$\alpha$ as compared to the unphosphorylated form. This observed reduction in activity then might be due to slow diffusion of the large $\beta$-subunit in the holoenzyme from the active site.

Proposed binding mode of protein phosphatase 2A (PP2A) and PKCK2$\alpha$

Ser/Thr phosphorylation is an important mode of transmitting the signal from one protein to another. Protein phosphatase carries out dephosphorylation of the phosphorylated proteins. They are classified into four main classes based on substrate specificity, sensitivity to inhibitors, subunit structure and cation requirements [37]. Type 2A phosphatases (PP2A) consist of catalytic ‘C’ subunit, a regulatory ‘A’ subunit (65 kDa) and one of the several ‘B’ regulatory subunits. PP2A is present as heterotrimeric ABC complex in most cell tissues. Catalytic C subunit is always found associated with regulatory A subunit. The three subunits are held together by extensive contacts between subunits [38]. PP2A is implicated in dephosphorylation of a number of proteins involved in cell proliferation and hence is an important regulator of cell growth.

It is well understood now that PP2A has a major role to play in deactivation of mitogen activated protein (MAP) kinase pathways. Phosphorylation of PP2A is a means of regulation of its activity. Heriche et al [13], Lebrin et al [14] and Cieslik et al [15] have reported the interaction of PP2A catalytic subunit (PP2Ac) with PKCK2. Lebrin et al and Heriche et al have shown that PP2Ac cannot bind to PKCK2 holoenzyme.
The human PP2A sequence was analyzed using PROSITE [37], PPSECRCH [http://www.ebi.ac.uk/ppsearch/ [39]] and NETPHOS [40] methods. These methods search in the given sequence a pattern that matches with the known sites of protein modifications such as phosphorylation by various kinases, acetylation, post translational modification and myristylation. They typically search for signature patterns in the query sequence. In figure 7, the alignment of PP2A with known phosphatase structures is shown indicating the positions of S/T-X-X-D/E motifs. Two positions with this sequence pattern have been observed in the PP2A. They are Thr 30 and Ser 201. A model of PP2A was generated and subjected to energy minimisation as discussed in Methods and the modelled structure is shown in figure 8. From the modeled structure, one finds that Ser 201 is buried due to the masking effect of 6-residue loop between the positions 212 and 217. Also Ser 201 is found to interact with main chain amide and carbonyl. These features are conserved in the homologous proteins of known structure used as templates in the comparative modelling. This is in accordance with the idea that buried polar charges have structural significance and are generally used to satisfy the main chain polarities. Hence in case PP2A is phosphorylated by PKCK2 Thr 30 (equivalent to Ser 22 in the α isofrom) that is exposed and present in an α-helical segment is a more likely candidate phospho acceptor site. Both isoforms, with Ser 22 as P site in α with Thr 30 as P site in case of β isoform can be accommodated at the substrate-binding site of PKCK2α. However in the current work we use the presence of putative PKCK2 phosphorylation sequence motif in PP2A only to identify the rough PKCK2α binding surface of PP2A and our complex model is independent of the phosphorylation or otherwise of PP2A by PKCK2.

Based on the model generated between 4-residue substrate peptide bound to PKCK2α as a template, a model for PP2Ac-PKCK2α interaction has been generated and is shown in figure 9. Many of the conserved residues of PKCK2α are involved in the binding to PP2Ac [18,19]. A model has also been generated (data not shown) with PP2Ac binding to the holoenzyme [PDB code: 1jwh, [19]]. Herriche et al [13] and Lebrin et al [14] reported that only PKCK2α can bind to PP2Ac. They hypothesize that the PKCK2β subunit when associated with the α-subunit as in the holoenzyme it may occlude the binding site of PP2A. PKCK2β and PP2Ac have severe steric clash when PP2Ac was force modelled onto the PKCK2 holoenzyme. The residues 22–98 of PKCK2β severely short contacts with residues 56–79 and 271 – 309 of PP2A. This could explain the experimental result of apparent displacement of PP2A when β-subunit binds to form holoenzyme.
Conclusions

PKCK2 is ubiquitous in eukaryotic cells. Although much is known about this kinase the mechanism of kinase-substrate interaction is not well understood. The signature sequence of phosphorylation by PKCK2, $S^*/T^*XXD/E$, is commonly found in several proteins and many of these may not be substrates of PKCK2. What factors drive the phosphorylation by PKCK2 of a subset of proteins with...
the signature sequence is not understood well. It appears from the current analysis that the driving force for binding with substrate proteins is charge and shape complementarity between two proteins apart from the suitable conformation and accessibility of the phosphorylation site. Most of the PKCK2 substrates present large acidic charges on their surfaces. However in the case of smaller peptide substrates the PKCK2 phosphorylation sequence motif in the right conformation and accessibility is the main requirement for binding as the problem of steric hindrance is expected to be not high due to the small size of the peptide substrates.

Charge complementarity of protein substrates also plays a leading role at least in some cases where substrates are phosphorylated only by holoenzyme and not PKCK2α, as in the case of HIV1 Rev protein. It is observed that the Rev protein is phosphorylated by PKCK2 only in the holoenzyme state [41–43]. This protein presents a cluster of positive charges downstream in the amino acid sequence of the PKCK2 phosphorylation site. This region is shown be responsible for favorable interactions with acidic stretches of PKCK2β [42]. The deletion of this basic stretch from HIV1 Rev protein, makes the mutant vulnerable to phosphorylation PKCK2α, which is not observed with wild type [42,43]. Polysine, a positively charged molecule known to stimulate the activity of PKCK2 holoenzyme for many substrates, is suggested to inhibit the phosphorylation efficiency of PKCK2 holoenzyme with Rev as substrate [42]. Polysine is known to bind to the β subunit of the holoenzyme [34,35]. Binding of a positively charged molecule might repel the approach of the Rev as substrate that is rich in Arg residues on its surface. The converse might be happening with calmodulin wherein the polysine might assist in binding of calmodulin to holoenzyme by charge complementation. Thus, β-subunit in the holoenzyme seems to be playing a role of providing favorable binding site to incoming substrates, thereby stabilizing the substrate-kinase interactions, which might be a factor in increased activity of the holoenzyme on many substrates. It is also reported that the α and β contacts are flexible [23]. The significance of this observation on PKCK2 holoenzyme activity is not known. The superposition of PKCK2α crystalized alone, and PKCK2α subunit from holoenzyme showed small but significant deviations in the N-terminal domain of the catalytic α subunit. Does these small structural variations result in increased activity of holoenzyme towards some of its substrates is an open question.

Holoenzyme formation is an important means of regulation of PKCK2α. In the holoenzyme state, the entry into the active site is not occluded completely, but, a protein substrate that presents with large surface of binding may not be able to thread into the substrate binding cleft, because of the steric restrictions imposed on the binding to active site. This is observed in the two of the proteins analysed here, calmodulin and catalytic subunit of PP2Ac. Once the substrate/binding protein and the PKCK2 catalytic subunit are bound effective association between the two molecules seems to be brought about by the specific interactions involving the basic helix of the kinase and the basic residues Arg 191 and Arg 195 at the carboxyl side of the kinase. It is not ruled out that PKCK2α would be able to accommodate substrates in non-turning conformations given substantial conformational change in the substrate presenting a turning conformation, which can not be reliably predicted by computational approaches. However, the observation and accommodation of helical regions in the PKCK2 binding site support the idea that PKCK2α can act on helical regions if no significant conformational changes happen at the kinase as well as at the substrate, as the substrate binds.

Based on the modelling studies it is also suggested that PKCK2 undergoes trans autophosphorylation with one copy of the β-subunit in a tetrameric holoenzyme molecular complex is phosphorylated by an α-subunit in a different molecular complex of the holoenzyme. An important outcome of this work is the suggestion that this trans autophosphorylation is reciprocal with the α-subunit in the first copy of the tetramer phosphorylating a β-subunit in the second copy of the tetramer.

**Methods**

**Modelling of substrate peptides bound to PKCK2α**

The consensus sequence pattern for phosphorylation by PKCK2 is S*/T*-X-X-D/E. This four residue peptide has been modelled on to the catalytic subunit of PKCK2 on the basis of the available crystal structures of different kinases complexed with inhibitor peptide that mimic the substrate. While modelling the substrate protein structure, in addition to the conformation of the consensus sequence pattern we have also used other influential factors like shape and charge complementarity. Interactive graphics softwares, SYBYL (Tripos Inc. St. Louis) and SETOR [44] have been used in modelling as well as the analysis of the complexed structures.

One of the crystal structures considered is cAMP dependent protein kinase bound to a peptide inhibitor [45]. The peptide has all the properties of the substrate of cAMP dependent PK, but the phosphorylatable serine residue, Ser21, is replaced by Ala. Hence although the peptide binds to the kinase, further phosphorylation or dissociation from the active site is not observed. Similarly, a ternary complex of phosphorylase kinase with non-hydrolyzable ATP analogue and substrate peptide is also considered for modeling substrates of PKCK2 on to the
enzyme [46]. The third structure considered is a ternary complex of the cyclin dependent kinase (CDK) complexed with non-hydrolyzable ATP analogue and substrate peptide [25]. PKCK2α, phosphorylase kinase, CDK and cAMP dependent kinase all adopt similar fold. Hence it is possible to superpose PKCK2α on to these known structures of kinases. This would bring the substrate peptide in the crystal structure at the substrate-binding site of PKCK2α. The superposition was done using the program SUPER (B.S. Neela, unpublished) by considering only those residues in the crystal structures that are involved in the recognition of the substrate and their topologically equivalent residues in the crystal structure of PKCK2α [24]. From this step, we generate a model of PKCK2α associated with a substrate peptide that is present in the crystal structure. The side chains of the P, P+1, P+2, and P+3 sites were then replaced by those found in the PKCK2α phosphorylation site. This ensures that the PKCK2 substrate peptide is in the same conformation as that seen in the crystal structure complex of another kinase. Further, specific peptide sequences that are identified as PKCK2 substrates have been modelled on the PKCK2 catalytic site using the structural model of PKCK2α and consensus phosphorylation sequence as the basis. It was ensured that there are no short contacts or other unfavourable structural features in the modeled complex structure. The model of PKCK2α bound to substrate peptide is then subjected to energy minimization as discussed below.

**Modeling of substrate or binding proteins bound to PKCK2α**

Examples of substrates/binding proteins to PKCK2 have been obtained from the literature [1,2,27]. A substrate protein or a binding protein of PKCK2 is considered for modelling as a complex with PKCK2 if the experimental structure of the substrate protein is available and the phosphorylation site (if substrate) is seen in the known structure. Calmodulin is one of the very few substrates of PKCK2 that is found in the Protein Data Bank (PDB) [47] with a crystal structure available [45]. Also the work of Meggio et al [30] has shown that calmodulin binds to PKCK2α only and cannot bind to the holoenzyme. The phosphorylation site of calmodulin is reported to be at Thr 79 and Ser 81 that are located at the central long helix and Ser 101 located in one of the globular domains in the structure. [29–31]. In the current work we have built a model of calmodulin-PKCK2α interaction corresponding to Ser 81 as the phosphorylation site on calmodulin as suggested by Marin, et al [31].

PKCK2β itself is a substrate of PKCK2α during the course of autophosphorylation [1,2,27]. We have also modelled PKCK2β bound to the α-subunit with Ser 2 as the phospho acceptor site in PKCK2β, following the method as discussed in previous section. Also, PKCK2β is known to be phosphorylated by the holoenzyme [49]. Hence a model of PKCK2β bound to holoenzyme was also generated.

**Comparative modelling of PP2A from known structures of Ser/Thr protein phosphatases**

Ser/Thr protein phosphatase 2A (PP2A) is modelled based on the template structures that were identified as close homologues from the PDB. There are two variants of PP2A, α and β isoforms, found in human. Each of these sequences is aligned with the sequence of known structure. The best alignment is between Protein Serine/Threonine Phosphatase-1 with resolution 2.1 Å [50] with sequence identity of 49% with α isoform of PP2A and 50% with β isoform of PP2A. The other structures that are similar to PP2A in sequence are PP2B calcineurin with 48% and 50% identity to α and β isoforms of PP2A, [51]. Two other complex structures of PP1A and PP2B are also available in PDB and they are complexed with okadaic acid in case of PP1A and FK506 in case of PP2B.

A three dimensional model of PP2A has been generated on the basis of crystal structure of PP1A, to which PP2A sequences show maximum similarity. A structure-based alignment of all known Ser/Thr phosphatase structures has been carried out using STAMP [52]. The structure-based sequence alignment has been made with PP2A sequences which were aligned using MALIGNS [53]. A 3-D model of PP2A has been generated using the suite of programs encoded in COMPOSER [54,55] and incorporated in SYBYL (Tripos Inc. St. Louis). The structurally conserved regions, which are largely helical and β-strand regions, in template structures are extrapolated to PP2A sequence. The rest of the regions that show high divergence from the sequence of the template structures sequence were modelled by identifying a suitable segment from a dataset of non-identical protein structures. This has been done by a template matching approach, wherein a search is made for the loop segments with required number of residues and that match with the end to end distances of the structurally conserved regions across the three `anchor’ Cα on either side of the loop. The hits so obtained are then ranked [56]. The best ranking loop with no short contact with the rest of the structure has been fitted using the ring closure procedure of F.Eisenmenger (unpublished results). Side chains are modelled on the equivalent positions as seen in template structure wherever appropriate or by using rules derived from analysis of known protein structures [57]. The model thus obtained was subject to energy minimization to relieve the short contacts if any. Energy minimization procedure adopted is discussed later in the section.
Prediction of PKCK2α interaction region in PP2A

Possibility of PKCK2α mediated phosphorylation of PP2A has been raised in the literature [13–15]. However, the proposed site of phosphorylation in PP2A by PKCK2α is not known to date. Here we locate the sequence motifs in PP2A consistent with PKCK2 phosphorylation solely to identify a rough PKCK2 binding surface of PP2A. Various pattern prediction programs PROSITE [39], PSERCH [http://www.ebi.ac.uk/ppsearch/ ] and [39] and NETPHOS [40] predicts Thr 30 and Ser 201 as the probable phosphorylation sites by PKCK2. We have analyzed each of these putative phosphorylation sites on PP2A models generated and docked onto PKCK2α with each of these sites oriented to be the phospho acceptor sites using the same principles as discussed previously.

Energy minimization

All the models generated have been subjected energy minimization using the AMBER force field [58] encoded in the SYBYL software. In the case of PP2A modelling, in the initial rounds of energy minimization, the backbone atoms were not allowed to move and the side chains alone were allowed to move in order to first sort out the short contacts amongst the side chain atoms. In the further rounds, the backbone atoms were also allowed to move. In the final cycles of minimization, electrostatics term has been included in the force-field. This approach ensured that the PP2A models generated are free of short contacts and bad geometry. Whenever energy minimization of complexes (containing two different polypeptide chains) is being carried out, it was ensured that the two molecules stay close to one another in an interacting distance. This has been done by applying a distance criterion, between two residues, one from each chain. The distance constraint has been imposed between the phospho acceptor Ser/Thr (Ser 1 in case of modelled four residue peptides, Ser 81 in case of calmodulin and Ser 2 in case of PKCK2β) and the catalytic base Asp 156 of PKCK2α such that they are maintained in an orientation and are in close proximity so that the hydroxyl group can accept the phosphate group from ATP.

Authors contributions

NR performed the computational sequence analysis and modelling. NS conceived of the study, and participated in its design and coordination. Both the authors read and approved the final manuscript.

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