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Edmond Fischer's kinase legacy: History of the Protein Kinase Inhibitor and PKA

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Summary:

Although Fischer's extraordinary career came to focus mostly on the protein phosphatases, after his co-discovery of Phosphorylase Kinase with Ed Krebs he was clearly intrigued not only by cAMP-dependent protein kinase (PKA) but also by the heat-stable high-affinity Protein Kinase Inhibitor (PKI). PKI is an intrinsically disordered protein that contains at its N-terminus a pseudo-substrate motif that binds synergistically with high-affinity to the PKA Catalytic (C) subunit. The sequencing and characterization of this inhibitor peptide (IP20) was validated by the structure of the PKA C-subunit solved first as a binary complex with IP20 and then as a ternary complex with ATP and 2 magnesium ions. A second motif, Nuclear Export Signal (NES), was later discovered in PKI. Both motifs correspond to amphipathic helices that convey high-affinity binding. The dynamic features of full-length PKI, recently captured by NMR, confirmed that the IP20 motif becomes dynamically and sequentially ordered only in the presence of the C-subunit. The Type I PKA Regulatory (R) subunits also contain a pseudo-substrate ATPMg2-dependent high-affinity inhibitor sequence. PKI and PKA, especially the Cβ subunit, are highly expressed in the brain, and PKI expression is also cell cycle-dependent. In addition, PKI is now linked to several cancers. The full biological importance of PKI and PKA signaling in the brain and their importance in cancer thus remains to be elucidated.

Keywords

Edmond Fischer; kinase; Protein Kinase inhibitor; Protein Kinase A; small Linear Motifs; pseudo-substrate

Introduction:

While Ed Fischer's career eventually shifted to the protein phosphatases after his initial discovery with Krebs of phosphorylase kinase (PhosK) as a regulator of the activity of glycogen phosphorylase activity [1–3], he nevertheless in the beginning retained a special fascination for cAMP-dependent protein kinase (PKA) and the novel high-affinity heat-stable inhibitor of PKA. His contributions to the chemistry and sequencing of both PKA

and PKI laid the foundation for the structural studies that followed more than 20 years after the discovery of PKA in 1968. The PKA C-subunit, the second protein kinase to be identified, was discovered by Fischer and Krebs as a contaminant of the PhosK prep because it phosphorylated and activated Phos Kinase [4]. It was initially called glycogen phosphorylase kinase kinase, so the concept of kinase cascades was demonstrated early on with those first two protein kinases. The heat-stable protein kinase inhibitor (PKI) was discovered shortly thereafter by Walsh, Fischer, Krebs and colleagues as a contaminant of the PKA C-subunit prep [5] and was quickly purified to homogeneity from both skeletal muscle [6] and brain [7]. Sequencing full-length PKI and localizing the inhibitor motif at the N-terminus of PKI was led a decade later by Scott, Fischer, Krebs, and Walsh in 1985 [8–10]. The discovery of a Nuclear Export Signal near the C-terminus of PKI came in 1995 [11]. We now consider PKI to be an Intrinsically Disordered Protein (IDP) that contains at least two Small Linear Motifs (SLiMs) - one that binds to the PKA C-subunit and the other to CREM1. Fischer also explored some early biology by micro-injecting the PKI inhibitor peptide into *Xenopus* oocytes, showing that this was sufficient to initiate cell division and maturation [12]. He was also one of the first to recognize tissue differences between PKI in skeletal muscle and the brain. His work with PKI thus demonstrates not only Fischer's biochemical expertise but also his creativity and his lifelong fascination with both biology and chemistry.

Discovery, purification, and characterization of the Heat-Stable Protein Kinase Inhibitor.

PKI was initially discovered very quickly after the PKA C-subunit was reported [4, 5]. It was a contaminant of the C-subunit prep that survived incubation at 95° and TCA precipitation, and it was quickly purified from both skeletal muscle and brain [6, 7]. Although the initial localization of the inhibitor sequence near the N-terminus of PKI was hypothesized by Demaille, Fischer and Walsh, it was three papers in 1985 that more definitively localized the inhibitory motifs of PKI. First was the PKI sequence by Scott, Fischer, and Krebs *et al*, which showed that PKI was a 75 amino acid protein [8]. Two subsequent papers localized the inhibitory region to the N-terminus [9, 10]. Scott *et al* identified the important arginines that resembled a substrate-like sequence as well as the predicted inhibitor sites in the PKA RI and RII regulatory subunits [9] and first identified PKI as a pseudo-substrate. Cheng *et al* focused also on the hydrophobic region that was N-terminal to the inhibitor sequence that was also important for high affinity binding [10]. These papers collectively defined PKI(5–24) or IP20 as a high-affinity inhibitor peptide.

The residue-specific characterization of these motifs was quantitatively analyzed with many synthetic peptides in 1987 by Glass *et al*. These studies rigorously defined the pseudo substrate motif that binds to the active site cleft [13] as well as a second hydrophobic region nucleated by Phe10 that was hypothesized to bind to a hydrophobic pocket that was near the active site [14]. In 1989 Reed *et al* predicted that the N-terminal region of IP20 containing Phe10 corresponded to an amphipathic helical motif [15]. Although it was shown in 1971 that PKI bound better in the presence of MgATP [5], the full magnitude of the enhanced binding of PKI in the presence of MgATP was not fully appreciated until the detailed kinetic characterization of PKI by Whitehouse and Walsh [16].

The IP20 binding site was thus defined and characterized before any structure was solved. It contained two functional motifs. The p−3 to p+1 residues (Arg-Arg-Asn-Ala-Ile) is the Inhibitor Site that includes the P-Site Ala (Table I). This motif was predicted to bind to the active site cleft, although this peptide does not bind with high affinity either in the presence or absence of ATP [17]. The PKI Inhibitor Site motif, which is a pseudo substrate, is analogous to the Kemptide, which is the canonical PKA peptide substrate derived from the PKA phosphorylation site in pyruvate kinase (Leu-Arg-Arg-Ala-Ser-Leu-Gly) [18]. The Km for Kemptide, is ~25 μM while the Kd is actually ~250 μM [18]. In contrast, the Km for PKI(5–24) in the absence of ATP is ~100 nM while in the presence of ATPMg it is 1 nM. The affinity for full-length PKI in the presence of ATP and Mg^{++} is sub nM [16, 17]. The binding of ATP is also synergistically enhanced by the binding of the PKI peptide (25 μM in the absence of IP20, which is similar to its Km in the Kemptide assay vs. 60 nM in the presence of IP20 [16].

Sequencing in Seattle.

It is important to appreciate the tremendous importance of protein sequencing in the early characterization of PKI and the PKA C-subunit. Fischer, who was trained as a chemist, always had an eye on the chemical properties of these proteins, and he was part of the team that sequenced both the PKA C-subunit [19] and PKI [8]. This team also published the sequence of several protein kinases during the early 1980s including Myosin Light Chain Kinase [20], Phosphorylase Kinase [21], and cGMP-dependent protein kinase [22]. This was all before cloning became routine, so these proteins were all sequenced manually, which was a long process that took several years. The University of Washington under the leadership of Hans Neurath was a Mecca for protein sequencing in the 1970s and 1980s.

The sequence of PKA, first sequenced manually and published in 1981, was extremely important because it was the first bona fide protein kinase to be sequenced. When Margaret Dayhoff, a pioneer in the emerging field of bioinformatics, saw this sequence, she immediately recognized the similarities to Src [23], which had been cloned in 1979 [24]. However, even though Src was demonstrated to be a protein kinase, when Src was cloned no one knew what a protein kinase should look like. Although the homologies with PKA were scattered in the Dayhoff alignment, they were sufficient to confirm unambiguously that PKA and the Src oncogene had evolved from a common origin. The protein kinase tree, later termed the Kinome [25], would thus be very large and would include not only the glycogen-related kinases and the other metabolic serine/threonine-specific kinases such as PKC and MLCK as well as the tyrosine-specific oncogenes that drive cancer. A few years later Hanks, Quin, and Hunter aligned 25 protein kinases, pre-blast, and, based on this alignment they identified 11 conserved motifs that were scattered throughout the kinase domain (Figure 1) [26]. This laid the foundation for the Kinome, which we now recognize to be one of the largest gene superfamilies. When the structure of PKA C-subunit was solved a few years later [27], these subdomains and their conserved motifs turned out to be a reliable description of the secondary structural elements of the conserved kinase domain.

The sequence of PKI, discussed above, was published in 1985 [8], and a second PKI isoform was later discovered as well as a second isoform of the PKA C-subunit [28–30]. PKIγ [31] and

several splice variants of PKIβ with broad tissue specificity added further to the complexity of PKI and its regulation of PKA signaling. The multiple isoforms of PKI are likely to be highly important for biological signaling, especially in the brain and in neurodevelopment. Additionally there is a potential correlation of PKI expression and prostate cancer [32, 33]. Thus it is fair to say that the full story of the PKI isoforms, including transfer into and out of the nucleus as discussed later, remains to be elucidated.

Fischer and his colleagues were also very interested in post-translational modifications. They were the first to identify the two phosphorylation sites in the PKA C-subunit [34] and others in Seattle identified the myristylation site on the N-terminus of the PKA C-subunit [35]. This was the first report of this novel acylation site that is added co-translationally. Myristylation is thought to be associated with anchoring to membranes, and we showed later, for example, that myristylation of the PKA C-subunit is sufficient to anchor the RIIβ holoenzyme to membranes [36]. Resh described the phospho-dependent myristylation switch in Src, and the PKA C-subunit also has a phosphorylation site (Ser10) near the N-terminus [37, 38].

Validation of IP20 binding site and functional sites in the PKA C-subunit. (Figure 2).

The first crystal structure of the PKA C-subunit that was co-crystallized with IP20 in 1991 built on all of the knowledge that had accumulated over the preceding two decades [39]. In parallel with the identification of IP20 as a high-affinity pseudo-substrate inhibitor, protein chemistry was used to characterize functional sites in the PKA C-subunit, and these experiments also validated some of the IP20 predictions. For example, in addition to the two endogenous phosphorylation sites in the PKA C-subunit there were two cysteines, one of which was very reactive, and, based on protection with ATP, this reactive Cys (Cys199), was hypothesized to be at the active site [40]. This prediction was validated when a Cys-containing variant of Kemptide was cross-linked to Cys199 [41]. Using affinity labeling coupled with protection by ATP, active site residues in the PKA C-subunit (Lys72, Glu91, and Asp184) were also identified [42, 43], and based on the Dayhoff and Quin alignments, these residues were all located in conserved motifs. These studies that identified functional motifs in the C-subunit thus complemented the extensive characterization of the inhibitor site in IP20. There was never any debate that we would crystallize the PKA C-subunit in the presence of IP20; this was a given.

So what did we learn from the crystal structure of the PKA C-Subunit? We discovered the protein kinase fold with its unique Glycine-rich loop. We added functional significance to the conserved motifs identified first by Dayhoff and then by Quin *et al* [23, 26]. We also identified the mechanism for binding an inhibitor/substrate site at the active site cleft as well as a distal hydrophobic tethering site that provided enhanced affinity for IP20. And we discovered the importance of two magnesium ions. Some of these things were appreciated at the time, but others were not. The crystal structure, however, completely validated all of the biochemical analyses of IP20 and the C-subunit. We summarize here what we learned and what we perhaps did not appreciate largely because we had this remarkable super stable pseudo-substrate inhibitor.

The C-subunit was first co-crystalized with IP20 as a binary complex in 1991 (Figure 2). This was the first crystal structure that was solved of a protein kinase, and the IP20 peptide that had been so well-characterized in the 1980s, contributed significantly to the success of the crystallization. That structure revealed the highly conserved bilobal structure of the protein kinase that is conserved in all protein kinases [27]. This was followed immediately by a second paper describing in detail the binding pockets that were occupied by IP20 [39]. This first structure completely confirmed the predicted importance of the arginine residues at the active site as well as the critical role of the hydrophobic residues that were distal to the active site and completely validated all of the extensive peptide studies. What this first structure did not contain, however, was the high-affinity binding site for ATP. The 1991 structure was a binary complex of the kinase and IP20, and although the binding pocket for ATP at the base of the cleft between the two lobes was surmised based on omit maps, the detailed features of the ATP binding site were not revealed until 1993 [44, 45]. To observe the binding of ATP one needed to have an excess of Mg^{++} while in the earlier structure there was an excess of ATP (1 mM) over Mg (0.5 mM). The structure obtained in 1993 with a 4-fold excess of Mg^{++} revealed not only the binding of the nucleotide but also the binding of two Mg ions, which had been predicted based on NMR by Armstrong and Mildvan [46]. The early work of Walsh showed that ATP enhanced the affinity of PKI binding to the C-subunit but only subsequently did we appreciate the magnitude of this synergy and the importance of the second Mg^{++} ion. McCammon in 2009 predicted computationally that the second Mg^{++} ion served as a “linchpin” metal that had to be released before ADP could be released [47] while Herberg and Doyle subsequently showed that the second metal ion was essential for the thermostability [48] and the high-affinity binding of ATP [49]. While this structure of the PKA C-subunit bound to IP20, ATP and 2 Mg^{++} ions (PDB ID: 1ATP) remains as the standard bearer for the protein kinase superfamily and is the structure that is used for homology modeling of other kinases, it is essential to also remember that this is a pseudo-substrate complex. PKI is a pseudo-substrate with an Ala at the P-site instead of a Ser/Thr. Do other kinases have similar high-affinity pseudo-substrate inhibitors or is this pseudo-substrate mechanism unique to PKA or at least amplified as a regulatory mechanism by PKA? This question remains to be answered, but in the case of PKA, where the C-subunit is synthesized as a fully active protein with a phosphate on the activation loop, we were very fortunate to have IP20, which locked the protein into a very stable and fully closed active conformation. The structure was deceptively simple; all the critical dynamics were yet to be discovered.

Description of the bi-functional IP20 Binding Site [39]:

The IP20 site is bi-functional. In addition to its arginine-rich pseudo-substrate site that docks to the active site cleft, it contains a distal amphipathic helical motif that conveys high affinity binding. The p−3 - p+1 inhibitor peptide docks into the active site cleft and three residues, the P+1 Ile and the P−2 and p−3 arginines are all essential binding determinants that stretch from the P+1 binding site in the C-lobe to the adenine binding site in the N-Lobe. The P+1 Ile binds to the hydrophobic pocket created by the P+1 Loop in the Activation Segment (Figure 2) while Tyr204 in the P+1 loop reaches over to Glu230 in the middle of the C-lobe. Glu230 is part of the binding site for the P−2 Arg that is positioned by Arg168 in the Catalytic Loop. Replacing Glu230 with Gln locks the C-subunit into a fully open

conformation that is unable to bind ATP [50]. In contrast replacing Tyr 204 with Ala prevents the transfer of the γ-PO4 of ATP to a protein/peptide substrate, although it is still capable of transferring the γ-PO4 to water [51, 52]. The p−3 Arg reaches up to the ribose ring of ATP. Most of the adenine binding residues come from the N-lobe while in the fully closed conformation the Phe327 and Tyr330 from the C-terminal tail are also anchored to the adenosine moiety of the ATP. The p−3 Arg thus touches all parts of the kinase domain and indirectly helps also to anchor the C-terminal tail into a fully closed conformation.

In addition to the active site pocket where the inhibitor peptide binds there is a distal tethering site that conveys the high-affinity binding of IP20. As predicted earlier, the N-terminal region of IP20 forms an amphipathic helix, which binds to a groove on the surface of the C-subunit. This binding site is mostly created by a motif between the αF and αG helices (residues 235–239; Tyr-Pro-Pro-Phe-Phe). The IP20 helix conveys high affinity binding (100 nM compared to 25 μM for a typical peptide substrate such as Kemptide) while in the presence of ATPMg the affinity is 1 nM. The importance of this hydrophobic site, and in particular Phe10, was predicted initially [14] and then confirmed by extensive peptide studies in 1989 where they also hypothesized that Phe10 was part of an amphipathic helix [15]. These predictions were all confirmed unambiguously by the 1991 and 1993 crystal structures [39, 44, 45].

Capturing the steps of catalysis in a crystal lattice with SP20.

IP20 can be converted into a substrate by simply replacing the P-site Ala with Ser. In this way, using a substrate mimetic of IP20 (Table 1), we were surprisingly able to trap all the steps of catalysis. To trap a substrate complex we used AMP-PNP, which was thought to be a non-hydrolyzable analog of ATP. Although Whitehouse and Walsh had shown earlier that AMP-PNP was not as effective as ATP in creating a high-affinity binding site [16], it was nevertheless presumed to be non-hydrolyzable. Surprisingly, when Bastidas crystallized this complex, he found after two months that he had captured the substrate complexes - AMP-PNP and PKS. However, this complex accounted for only 45% of the crystals. The other 55% corresponded to the product complex - ADP and p-PKS. After six months of crystallization, no substrate complexes were left only product [53]. In addition, he found that after 6 months one of the Mg ions lacked density, which resolved an earlier prediction of a “linchpin” Mg ion that needed to be removed before ADP could be released [47]. Eventually all the steps of catalysis were captured in a crystal lattice with the PKS variant of the PKI peptide.

PKI is an Intrinsically Disordered Protein/Peptide that also contains a Nuclear Export Signal (NES).

We now recognize PKI to be an Intrinsically Disordered Peptide/Protein (IDP) although that terminology did not exist when PKI was discovered [54]. Although IDPs are mostly disordered in solution and are computationally identified as disordered [55], small Linear Motifs (SLiMs) that target the IDP to different binding partners are typically embedded within the IDP. IP20 contains two SLiMs (Figure 3). The first SLiM is actually bi-functional. It contains the highly charged Inhibitor site, which is completely disordered in solution and an N-terminal hydrophobic region. Based on NMR and MD simulations this N-terminal region, which was

predicted to have an amphipathic helix [15], has a propensity to form a helix as well as a beta turn in solution [56] (Figure 4, center). Both sites in IP20 are required for inhibition of the PKA C-subunit, and in the presence of the PKA C-subunit both become well-ordered. The inhibitor peptide docks into the active site cleft between the N- and C-lobes while the amphipathic helix is anchored to a hydrophobic groove in the C-Lobe and contributes to high affinity. Both are even more tightly anchored when ATP and Mg^{++} are present (Figure 4).

In 1995 we identified another SLiM further down in the PKI sequence that corresponded to a Nuclear Export Sequence (NES). The NES was predicted to be an amphipathic helix (Figure 3) [57]. While basic Nuclear Localization Sites (NLSs) had been well-studied at this time [58], the existence of an NES was contested. The discovery of an NES in PKI and HIV-Rev1 [59] marked a turning point in nuclear transport, and this NES in PKI remains the strongest of all the NES motifs that have subsequently been identified [60]. Like the PKI inhibitor site, based on time-resolved fluorescence anisotropy, docking of the NES required the hydrophobic surface of an amphipathic helix [61] (Figure 4). The NES amphipathic helix binds to CRM1:Ran(GTP), and the structure of this complex was eventually solved in 2010 [62] (Figure 5). With this complex we have two biological switches - a GTPase bound to GTP and a kinase domain bound with high affinity to PKI. The mechanism for the translocation of this complex out of the nucleus and the putative release of the C-subunit in the cytoplasm remains to be elucidated. The expression of PKI is also regulated in a cell cycle-specific manner [11]. So there are still many features of PKI functioning in cells that remain to be elucidated.

Capturing the dynamic features of full-length PKI.

Shape conformers that were described early on suggested that plasticity was an intrinsic feature of PKI [63]. and some of this was captured using NMR for IP20 [56] and Fluorescence Resonance Energy Transfer (FRET) for full-length PKI, which showed that the N-terminal helix is transient in the unbound IP20 while the inhibitor site is only stable when the peptide is bound to the C-subunit [61]. However, Veglia and his colleagues recently captured the dynamic interactions of full-length PKI with the PKA C-subunit using NMR, SAXS, fluorescence, meta-dynamics, and Markov State modeling [64]. They first confirmed PKI was highly disordered, with two transient helices, one in the high-affinity region and the other in the NES binding region. By then comparing free PKI with PKI bound to the C-subunit, they elegantly describe the highly dynamic nature of PKI as it binds to the PKA C-subunit and poises itself for recognition of CRM1:Ran(GTP) (Figure 5, left and right panels). The binding kinetics revealed a fast interaction between PKI and C-subunit, which was mediated by the arginine-rich Inhibitor Site, and a slower conformational rearrangement attributed to the binding of the N-terminal helix and probably the P+1 Ile. This work also provides a mechanistic model showing how the ordering of the IP20 region poises the C:PKI complex for binding to CRM1:Ran(GTP). By capturing the communication that takes place between the C-subunit and the region following the inhibitor site, they depict a highly dynamic portrait of the complex allosteric cross-talk that is mediated by the intrinsically disordered PKI. Obviously the next steps will be to characterize PKI-mediated complexes that contain both the PKA C-subunit and CRM1:Ran(GTP). This complex captures two of the most

important switches in biology - a kinase and a GTPase - and nucleotide binding is critical for both.

Pseuodsubstrate motifs are embedded in the PKA Type I Regulatory Subunits.

Is inhibition by high-affinity pseudo-substrate inhibitors like the PKI isoforms a unique feature of PKA signaling, or do other protein kinases also have small pseudo-substrate inhibitors? While such pseudo-substrate inhibitors may be unusual for other kinases, for PKA there is another potentially even more important pseudo-substrate inhibitor. In addition to PKI, the RI subunits (RIα and RIβ) of PKA have a pseudo-substrate motif in contrast to the RII subunits (RIIα and RIIβ), which are bona-fide PKA substrates (Table I). In all cases, these inhibitor proteins need distal tethering sites far from the active site cleft in order to achieve high-affinity binding. The difference is that in the case of pseudo-substrates, $ATP(Mg)_2$ is required to achieve high-affinity binding while substrate inhibitors like RIIα/β bind with high-affinity independent of ATP [65–68]. The RIα subunit can be captured in a synergistic high-affinity complex with the C-subunit similar to PKI; however, in this case the R-subunits use another different high-affinity tethering site that is occupied the first cyclic nucleotide-binding domain (CNB-A). In contrast to PKI where the inhibitory helix is N-terminal to the Inhibitor Site, the CNB-A domain lies C-terminal to the inhibitor site and docks onto the surfaced that is formed by the outward-facing residues of the Activation Segment (Figure 6). Like PKI the most stable form of the RIα holoenzyme requires the presence of ATP and 2 Mg^{++} ions where the affinity of the C-subunit for RIα is sub nM [47]. High-affinity binding of RIα to the C-subunit also requires the two arginines in the inhibitor site [69]. In contrast, in the absence of ATP and 2 Mg^{++} ions, the affinity is ~100 nM. This binding affinity is still within a physiologically-relevant range and is probably even more important when the IC50 for activation by cAMP is ~60–100 nM. The RIα holoenzyme likely toggles between these two states where $ATP(Mg)_2$ and cAMP are competing for control [68, 70]. In contrast to RIα, the RII subunits form a sub nM complex in the absence of ATP, but they need another distal tethering site to achieve high-affinity binding (Figure 6), which is contributed by the CNB-B domain [71]. In the case of Type II holoenzyme, the two arginines in the inhibitor site are not required; instead, in the absence of P−2 and p−3 arginines, a constitutively active holoenzyme is formed [72].

Future challenges.

What about the role of PKI in biology? Even more importantly how does PKI expression and localization correlate with disease? While there are still many questions related to PKI that need to be addressed at the structure and mechanism level, perhaps the most critical unanswered questions relate to the role that PKI plays is *in vivo* signaling in tissues, especially in the brain, where both PKI isoforms and PKA are highly expressed (Figure 7) [73, 74]. The importance of PKA signaling in the brain has been emphasized since the discovery of PKA in 1968 [4] and the high abundance of PKI in the brain was cited in the first PKI paper [5], The early work of Greengard, Nathanson and others [75, 76] was one of the reasons that led Fischer to purify PKI from the brain in 1978 [7]. Although PKA signaling has been linked to neurodevelopment, neurotransmission, gene transcription, and synaptic plasticity for decades, the detailed mechanisms still remain unclear and only recently have we begun to appreciate the importance of isoform specificity, both in PKA and PKI.

Shortly after the discovery of the NES in PKI, using PKIα-specific antibodies, we showed that the expression and localization of endogenous PKI is highly regulated as a function of the cell cycle in thymocytes and in REF52 cells [11], but the details of this observation, as well as potential isoform-specific differences, have yet to be elucidated. With regard to tissue specificity, especially in brain, RNA hybridization suggested that isoform-specific localization will be important [31]. The importance of PKI in neuroplasticity was also demonstrated in rat hippocampal sections where PKI is specifically enriched in the dentate gyrus cells (Figure 8, right). Several types of synaptic stimulation of rat hippocampus including electrolyte stimulation and stimulation with kainic acid showed a marked decrease in PKI in the dentate granule cells, which was tracked both by mRNA hybridization and Immunochemistry [77]. Antisense oligonucleotides also led to reduced excitability, further supporting the hypothesis that PKI may contribute significantly to synaptic activity.

While early PKI knockout experiments did not show a strong phenotype [78, 79], similar experiments showed that RIβ and RIIβ were linked specifically to disruptions in neuronal function [80–82]. Depletion of RIβ, in particular, led to learning deficiencies and a loss of Long Term Potentiation (LTP). High-Resolution Mosaic Imaging of RIβ and RIIβ in the hippocampus revealed distinct patterns of localization, which was consistent with the phenotype (Figure 7, left) [83], but it is not clear how this correlates with the imaging of PKI. Obviously, we need to further characterize the functional role and localization of these PKA and PKI isoforms.

Cβ isoforms in brain.

The importance of PKA isoforms, especially in the brain, has also been relatively unexplored. It is also interesting and important that a second form of the PKA C-subunit, Cβ, is also abundantly expressed in neurons [80]. As much as 50% of PKA signaling in the brain is likely mediated by Cβ isoforms, including several N-terminal splice variants [74]. Using the retina as a window into the brain we showed with isoform-specific antibodies that Cα and Cβ localized differently and that Cβ selectively localized to mitochondria [80]. We did not, however, screen for PKI isoforms in the retina. As indicated above, PKI isoforms are also highly expressed in the brain, especially PKIα, and based on RNA hybridization and imaging with isoform-specific antibodies, they are localized very specifically. Thus it is essential to use cell imaging to define PKA signaling, in parallel with high resolution studies, which tell us about structure and dynamics at high resolution. It is interesting that RIβ and PKI are both localized and enriched in the hippocampus but whether they co-localizes to the same cells is not known. Cα and Cβ also need to be imaged in mouse brain. Just as we need to elucidate the role of PKI in regulating or influencing the cell cycle, it will eventually also be critical to know how PKA and PKI signaling contribute to neuronal functions.

PKI and disease.

And what about PKI and disease? PKA signaling is associated with cardiomyocyte hypertrophy [84], metabolic disorders [85], cancer [86, 87], neuropotentiation [77] and potentially with neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease [70]; however, growing evidence suggests that PKI may also be linked to cancer in very cell-

specific ways. In skin cells, for example, expression of IP20 led to tumor growth suggesting that PKA may serve as a tumor suppressor [87]. More recently the expression of PKI was shown to be elevated in many cancer cells and in this case, using activation of MAPK as a downstream readout of PKA signaling and tumor growth, expression of IP20 led to a reduction in tumor growth [32]. Inhibition of PKA by the IP20 peptide showed enhanced survival and increased MAPK activation. These studies suggest that we still have much to learn about PKI and its role in biology and disease. In addition to transgenic animal where IP20 is expressed in a tissue-specific manner therapeutic reagents that target these sites are being developed. An example is the stapled peptide version of IP20 that penetrates cells and has a low nM affinity [88]. This represents a new frontier in PKA signaling in which PKI is likely to play a central role.

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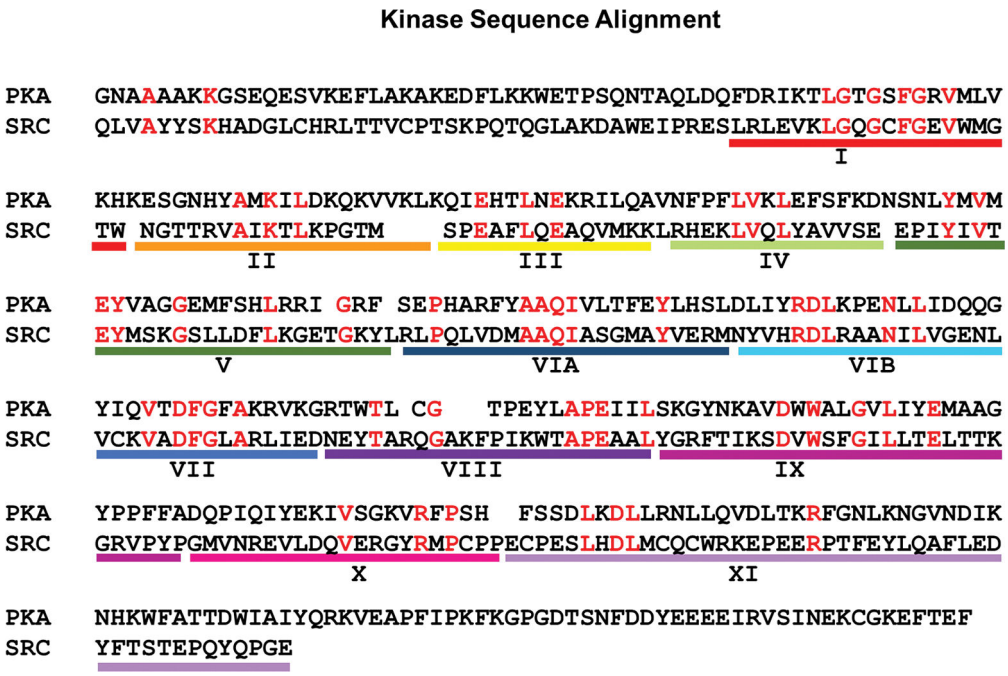

Figure 1. Alignment of conserved motifs in the protein kinase superfamily.

The sequence of PKA, published in 1981 [19] was aligned with the cloned sequence of Src [23]. This alignment confirmed that Src and the PKA C-subunit had evolved from a common precursor and established unambiguously that Src and PKA belonged to the same gene family. Based on the alignment of 25 protein kinases, Quin *et al* aligned 25 protein kinase sequences and identified a set of 11 sub-domains that each contained conserved motifs [26].

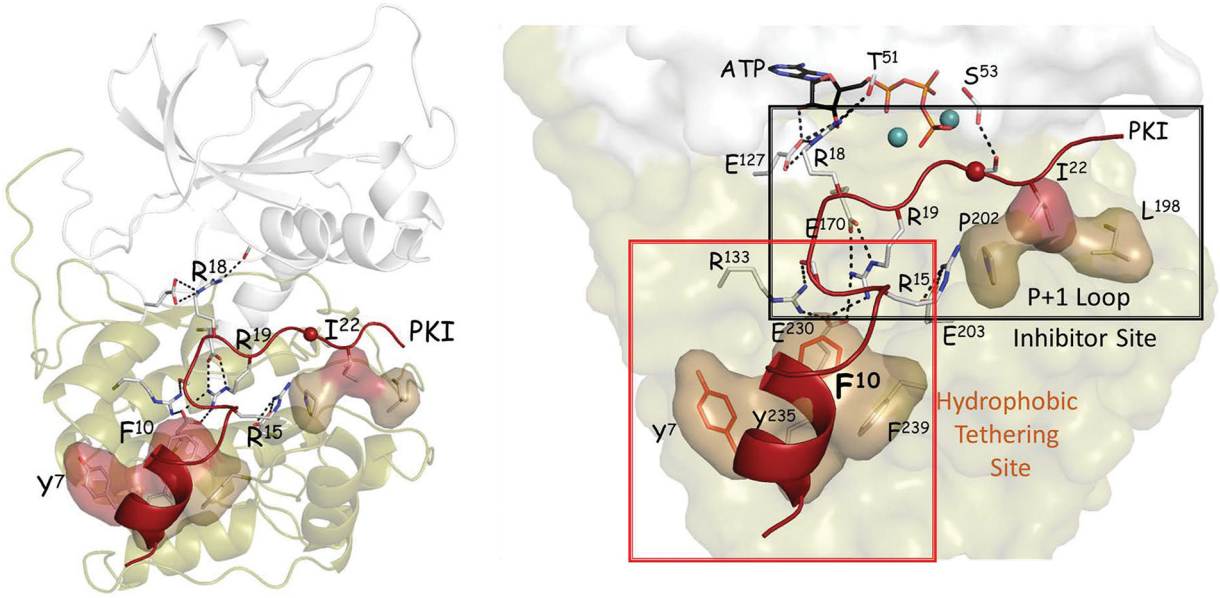

Figure 2. Structures of the PKA C-subunit bound to IP20 and ATP.

The first PKA structure solved in 1991 was a binary complex (left panel) that contained the PKA C-subunit and IP20 [27, 39]. When excess Mg^{++} was added, the ternary complex was obtained [44]. The p−3 to p+1 Inhibitor Site reaches across the active site cleft (black box) while the distal amphipathic helix docks onto a hydrophobic surface of the C-subunit as a hydrophobic tethering site (red box).

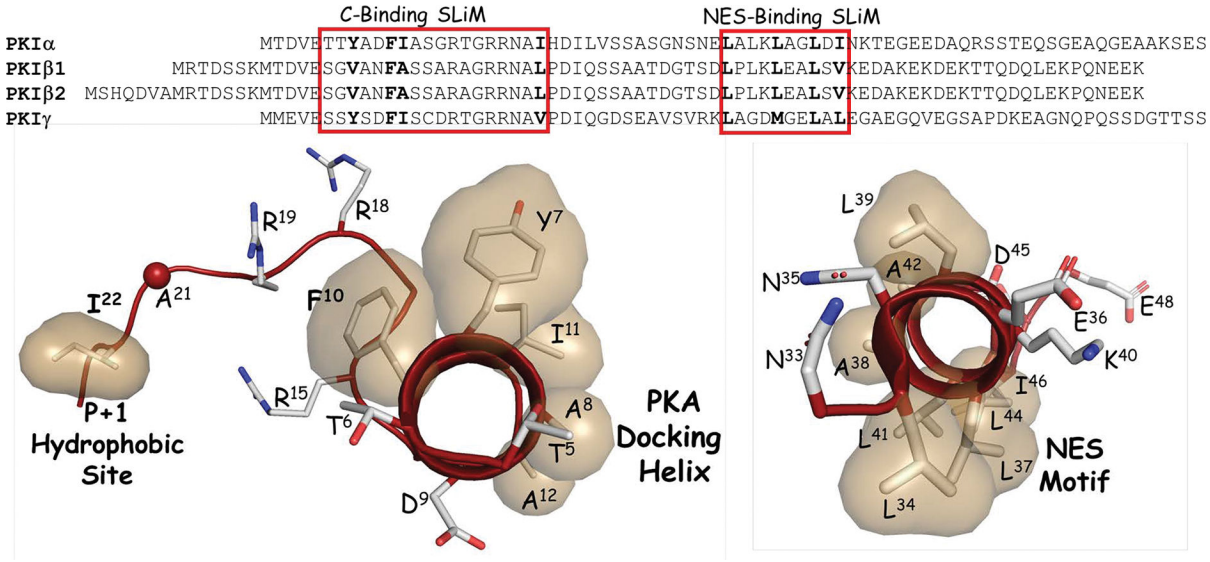

Figure 3. Small Linear Motifs embedded in PKI.

There are several Small Linear Motifs (SLiMs) embedded in the sequences of the three PKI isoforms. The first motif that docks onto the PKA C-subunit is bi-functional and includes the amphipathic helix, a beta-turn, and the Inhibitor sequences as indicated in Table 1. The second SLiM corresponds to an amphipathic helix that docks onto CRM1:Ran(GTP) [57, 62].

Figure 4. Capturing Order/Disorder transitions of PKI.

The middle panel, based on NMR [56], captures the disorder of the inhibitor site of IP20 in the absence of the PKA C-subunit, while in the presence of the PKA C-subunits and $ATP(Mg)_2$ the inhibitor site is docked into the active site cleft of the bilobal C-subunit (white ribbon). In contrast the helix, which is partially ordered in solution, is firmly anchored to a hydrophobic pocket on the surface of the PKA C-subunit. On the left and right are models of the length PKI in the absence (left) and presence (right) of the PKA C-subunit. These models best represent the SAXS data. The overall ordered docking of full-length PKI, based on NMR, SAXS, fluorescence, meta-dynamics and Markov State modeling, provides our first mechanistic model for the dynamic docking process and also shows how the region following the Inhibitor Site is not only primed for recognition of CRM1 but also how it mediates allosteric cross-talk with the C-lobe of the PKR C-subunit [64].

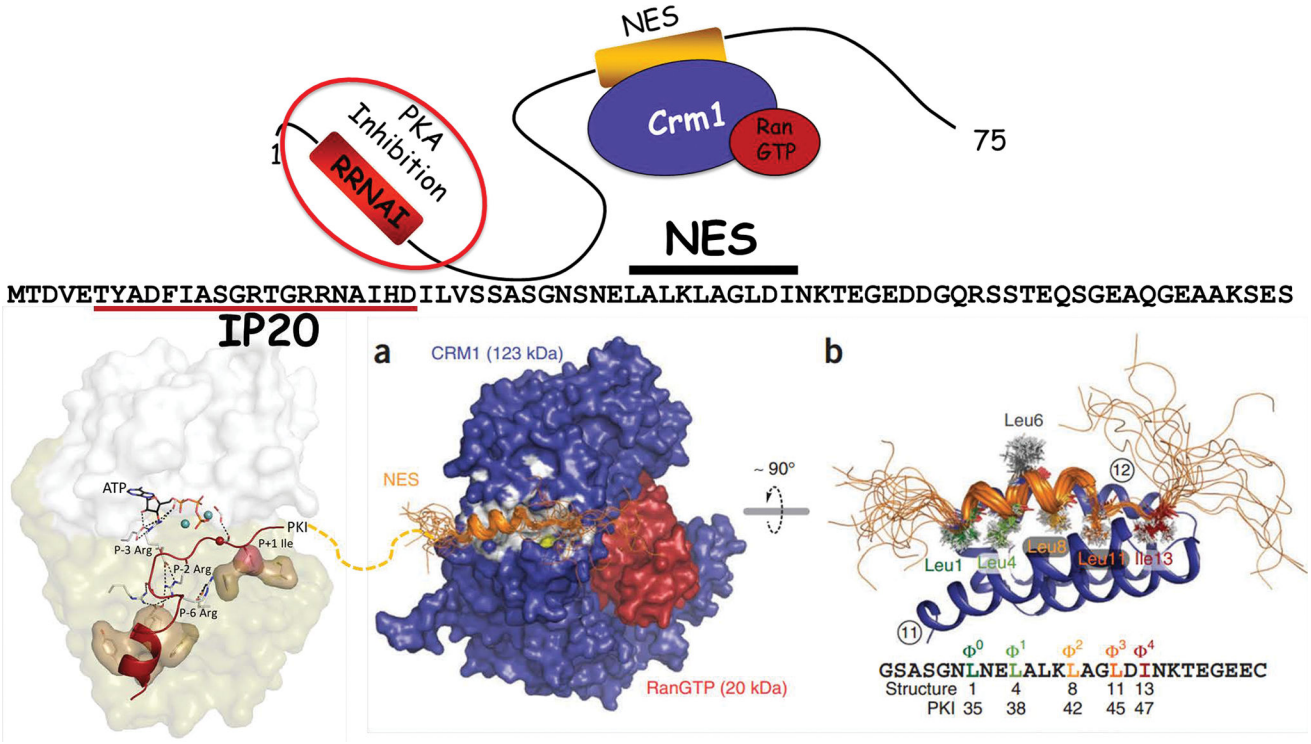

Figure 5. Binding of the PKA C-subunit and CREM1:Ran(GTP) to PKI.

The two Slim motifs in PKI bind with high-affinity to the PKA C-subunit (PDB:1ATP) and CRM1:Ran(GTP) (PDB:3NBY). In both cases high-affinity binding is mediated by the hydrophobic surface of an amphipathic helix.

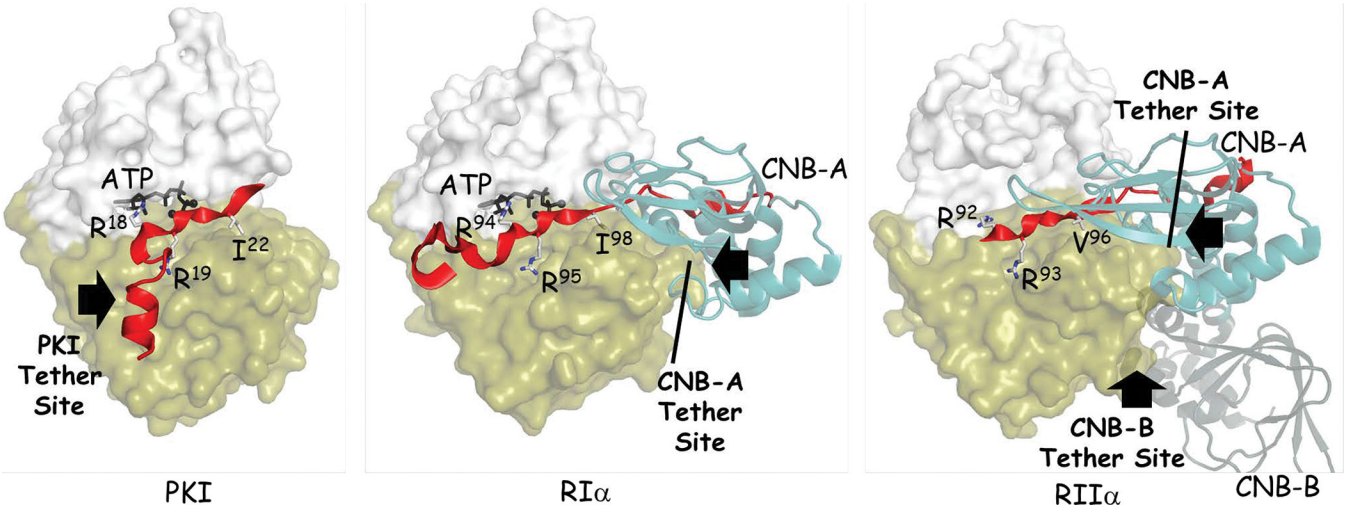

Figure 6. Distal tethering sites on the C-Lobe of the PKA C-subunit trap high affinity inhibitor proteins.

All PKA inhibitor proteins, PKI isoforms and R-subunits, share a common Inhibitor Site. PKI (left) and RI-subunit (middle) are pseudo-substrates and require $ATP(Mg)_2$, while RII-subunits are substrates and require only the two cyclic nucleotide binding (CNB) domain tethering sites (CNB-A and CNB-B) to achieve high-affinity binding. Left panel: High-affinity binding of IP20 requires the amphipathic helix containing Phe10 that lies N-terminal to the inhibitor site while the Inhibitor site is trapped by ATP(Mg)2 [44]. The middle panel shows how the CNB-A domain of RIα is docked onto the C-lobe of the kinase via a different distal tethering site while the inhibitor site is trapped by $ATP(Mg)_2$[89]. In this case high-affinity binding also requires the region that lies N-terminal to the Inhibitor site, but it is distinct from the PKI tethering site. The right panel shows how the RIIα subunit docks onto the CNB-A tethering site and onto the CNB-B tethering site and this is sufficient to convey high-affinity binding in the absence of ATP [71].

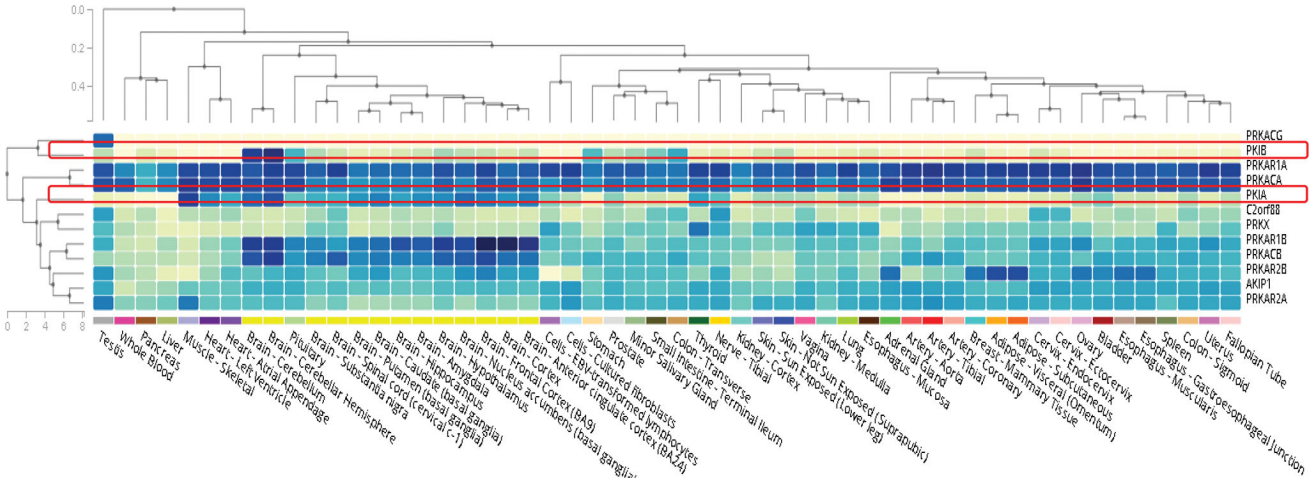

Figure 7. Tissue-specific expression of PKA and PKI isoforms.

The expression taken from the GEXT web server shows that PKIα is highly enriched in the brain. Cα (PRKACA), RIα (PRKAR1A), and RIIα (PRKAR2A) are expressed constitutively at high levels in most cells while the expression of Cβ (PRKACB), RIβ (PRKAR1B), and RIIβ (PRKAR2B) are all enriched in neuronal tissues.

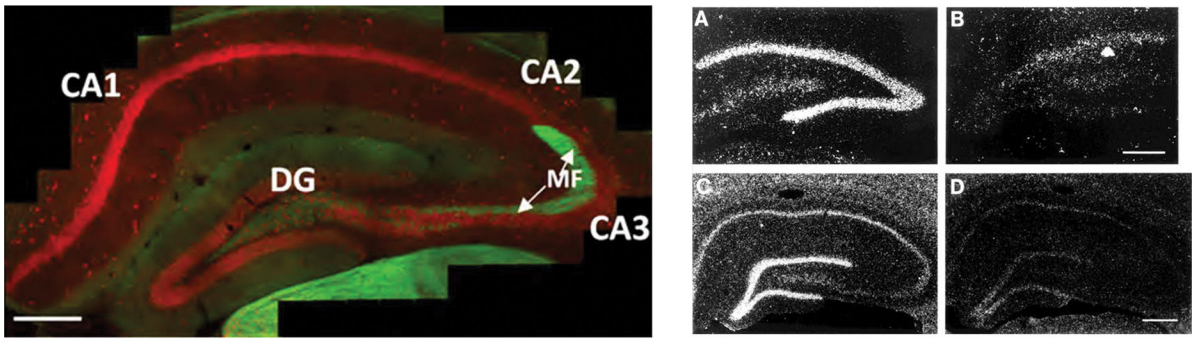

Figure 8. Imaging of endogenous PKI and PKA subunits.

Left panel: High-Resolution Mosaic Imaging of RIβ (red) and RIIβ (green) in mouse hippocampus using isoform-specific antibodies [83]. Right Panel: Imaging of PKIα mRNA in dentate gyrus region of rat hippocampus [77]. Panels A and C compare the hemisphere that had been injected with kainic acid to stimulate synaptic activity (C) with the untreated hemisphere (A). Panels B and D compare electrolytic lesion of the hilius, which is known to stimulate synaptic input to granule cells.

Table I.

Substrate and pseudo-substrate sequences that bind to the active site of the PKA C-subunit.

	Amphipathic Helix	β-turn	Inhibitor Site
IP20:	TT**Y**AD**F**IA	SG**R**T	G**RR**N**A**I**H**D
SP20:	TT**Y**AD**F**IA	SG**R**T	G**RR**N**S**I**H**D
Kemptide:			L**RR**A**S**L**G**
RIα:	SPPPPNP	VVKGR	R**RR**G**A**I**S**
RIβ:	SPTPPNP	VVKGR	R**RR**G**G**V**S**
RIIα:	DADLEVP	VPSKF	T**RR**G**S**V**C**
RIIβ:	AGAFNAP	VINRF	T**RR**A**S**V**C**

All share a common Inhibitor Site that extends from the p-3 site to the p+1 site as indicated by the black box. The arginines are highly conserved as is a hydrophobic residue at the p+1 site. In PKA substrate the P-Site is either a Ser or Thr. The amphipathic helix in PKI that docks onto a hydrophobic pocket of the C-lobe is enclosed in the red box.