

Review article:

TARGETING PROTEIN KINASE A IN CANCER THERAPY: AN UPDATE

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ABSTRACT

Protein Kinase A (PKA) is a well known member of the serine-threonine protein kinase superfamily. PKA, also known as cAMP-dependent protein kinase, is a multi-unit protein kinase that mediates signal transduction of G-protein coupled receptors through its activation upon cAMP binding. The widespread expression of PKA subunit genes, and the myriad of mechanisms by which cAMP is regulated within a cell suggest that PKA signaling is one of extreme importance to cellular function. It is involved in the control of a wide variety of cellular processes from metabolism to ion channel activation, cell growth and differentiation, gene expression and apoptosis. Importantly, since it has been implicated in the initiation and progression of many tumors, PKA has been proposed as a novel biomarker for cancer detection, and as a potential molecular target for cancer therapy. Here, we highlight some features of cAMP/PKA signaling that are relevant to cancer biology and present an update on targeting PKA in cancer therapy.

Keywords: PKA, cAMP, cancer therapy, designing kinase inhibitors

THE cAMP-DEPENDENT SIGNALING PATHWAY AND ITS EFFECTORS: AN OVERVIEW

Adenosine 3'5'-cyclic monophosphate (cyclic AMP, cAMP) was first identified as a small intracellular heat-stable factor mediating the effect of glucagon on the phosphorylation status of glycogen phosphorylase in the 1950s, and the concept of cAMP as an important mediator for many extracellular signaling molecules was rapidly developed (Beavo and Brunton, 2002).

cAMP is present in every cell, where it is generated from ATP by adenylate cyclases, ACs, and can be induced more than twenty-fold upon activation of ACs by extracellular signals (Hanoune and Defer, 2001). Degrada-

tion of cAMP is mediated by cAMP phosphodiesterases, PDEs, that hydrolyze cAMP into adenosine 5'-monophosphate and this event is important for controlling cAMP resting state levels (Omori and Kotera, 2007).

So, intracellular concentration of cAMP results from the fine balance between the activities of synthesis and degradation by adenylate cyclases and cAMP phosphodiesterases, respectively.

A large number of hormones, neurotransmitters and other signal substances utilize cAMP as an intracellular second messenger, so that the rate of cAMP production and degradation is sensitive to a wide range of extracellular and intracellular signals, and cAMP can directly regulate a variety of cell

functions (Gancedo, 2013). Within each cell, cAMP may activate different proteins. For example cAMP may operate directly on ion channels (Biel, 2009).

An important additional effector system for cAMP signaling is achieved by the exchange proteins directly activated by cAMP 1 and 2, Epac1 and Epac2, also named cAMP-GEFI and -II. These guanine nucleotide exchange factors (GEFs) are specific activators of the small GTPase Rap1 (Schmidt et al., 2013). The cAMP-binding domain of Epac can bind one molecule of cAMP, resulting in a conformational change of the protein, which will expose the active site of the catalytic domain, enabling the protein to bind to and activate Rap1 (De Rooij et al., 1998).

However, the main intracellular target for cAMP in mammalian cells is the cAMP-dependent protein kinase (PKA; EC 2.7.1.37) we will discuss on extensively below (Taskén et al., 1997).

cAMP, either via a PKA-dependent or PKA-independent manner, affects numerous cellular functions such as metabolism, ion channel activation, cell growth and differentiation, gene expression and apoptosis (Antoni, 2012).

On the other hand, the cAMP pathway interacts with other intracellular signaling pathways, including cytokine and Ras-Raf-Erk pathways (Yu et al., 2013; Kostenko et al., 2011; Spina et al., 2013; Follin-Arbelet et al., 2013; Tai et al., 2014).

Notably, these signaling connections play an important role in cancer biology and a combined blockade of such signaling pathways is considered a relevant strategy for therapeutic intervention (Awada and Aftimos, 2013; Colzani et al., 2014).

The existence of different cAMP downstream effectors and some features of PKA signaling pathway may contribute to explain how differential discrete effects of cAMP may be obtained (Skalhegg and Tasken, 2000).

An important concept is that cAMP concentration and cAMP signaling can change

and occur very locally, respectively (Edwards et al., 2012; Lefkimmiatis and Zaccolo, 2014).

Localized cAMP-mediated activity is explained by localized induction and degradation of cAMP by PDEs in specialized cellular compartments such as caveolae and lipid rafts (Simons and Ikonen, 1997). Also ACs and GPCRs are not evenly distributed along the membrane (Willoughby and Cooper, 2007; Steinberg and Brunton, 2001).

Noteworthy, in the same cell, elevation of cAMP and subsequent PKA activity by different agonists can lead to different physiological responses, even because receptors for extracellular signaling molecules can activate only a fraction of PKA that are largely segregated in subcellular microdomains by a great number of PKA Anchoring Proteins, AKAPs (Beene and Scott, 2007). AKAPs are bound to cytoskeletal proteins or organelles and bind regulatory subunits of the PKA, so that the PKA can be docked and concentrated close to crucial targets and, despite their broad substrate specificity, can phosphorylate only selected proteins (Gold et al., 2006).

This all contributes to a localized activation of PKA.

PKA: GENERAL FEATURES, SIGNALING AND CANCER

Protein Kinase A isozymes: Features of regulatory and catalytic subunits

A major function of cAMP in eukaryotes is the activation of PKA. cAMP acts in mammalian cells by binding to two distinct isoforms of PKA, defined PKA-I and PKA-II. PKA-I and PKA-II differ in regulatory (R) subunits, termed RI in PKA-I and RII in PKA-II, respectively. PKA holoenzymes are inactive heterotetramers. Binding of two cAMP molecules to each of the regulatory subunits results in the release and activation of the catalytic subunits. These catalytic subunits will phosphorylate serine and threonine residues on specific substrate proteins both in the cytoplasm and in the nucleus (Skalhegg and Tasken, 2000).

The major nuclear targets of PKA are the transcription factors of the cAMP response element binding (CREB) family (Mayr and Montminy, 2001). CREB proteins bind optimally to palindromic CREs (sequence TGACGTCA) in promoters and upon phosphorylation by PKA they recruit the coactivator, CREB binding protein (CBP) to the promoter. Such a phosphorylation event results in the induction of cellular gene expression (Sands and Palmer, 2008). The importance of CREB for several physiological events has been confirmed by the high number of targets identified so far: up to 4000 genes involved in several cellular processes. Moreover, several lines of evidence obtained from studies on leukemia, fusion oncoproteins, viral oncoproteins and endocrine tumors support the notion that CREB is involved in oncogenesis (Siu and Jin, 2007; Sakamoto and Frank, 2009).

The regulatory subunits are highly dynamic multi-domain proteins that interact with a variety of proteins in addition to serving as major receptors for cAMP (Kim et al., 2007; Wu et al., 2007). Although there are multiple isoforms (I α and I β , II α and II β), all retain the same general architectural organization. All have a dimerization/docking (D/D) domain at the N-terminus, which is the docking site for the A kinase-anchoring proteins, AKAPs (Gold et al., 2006). The D/D domain is followed by an inhibitor site (a pseudosubstrate for RI subunits and a substrate site for RII subunits) and two cAMP binding domains (CBDs), referred to here as domains A and B. Structures of the cAMP bound conformations of RI α and RII β revealed that the CBDs were conserved motifs that resemble the catabolite gene activator protein (CAP) in bacteria (Su et al., 1995; Diller et al., 2001; Kim et al., 2005). Different subunit isoforms (RI α , RI β , RII α , and RII β) have different affinities for cAMP, thus originating holoenzymes (PKA type I or PKA type II with different subunit composition and affinity for cAMP and thus are activated at either low or high local concentrations of cAMP in the cell (Skalhegg and

Tasken, 2000). Typically PKA type I holoenzyme have higher affinity for cAMP than type II holoenzyme. When RI subunits are up-regulated, cAMP sensitivity of PKA increases and thereby lowers the threshold for activation of cAMP-mediated downstream effects. Furthermore, only PKA-II but not PKA-I undergoes autophosphorylation (Rosen and Erlichman, 1975), which serves as a “feed-forward” signal by enhancing the cAMP responsiveness of PKA-II (Martin et al., 2007).

The four types of regulatory subunits have different expression patterns in mammals (Skalhegg and Tasken, 2000). Moreover, during both physiological and pathological conditions, the composition of the PKA holoenzyme as well as their intracellular localization may change, inducing different effects. While RI α has ubiquitous distribution, RI β is expressed primarily in brain, testis and B- and T-lymphocytes. Similarly, RII α has ubiquitous distribution, while RII β is expressed in brain, adipose, and some endocrine tissues. Besides cAMP affinity, localization of the holoenzyme is also different, with PKA type I enzymes being generally cytoplasmic, and type II enzymes specifically anchored to subcellular structures and compartments. The AKAPs play an important role in differential targeting of PKA types I and II in the cell. The RI α and RI β subunits are dissimilar, but reveal high homology (81 % identity at the amino acid level) as do the RII α and RII β subunits (68 % identity at the amino acid level). Importantly, as reported below in “PKA signaling and cancer” section, PKA type I is associated with growth and proliferation whereas PKA type II is associated with increased differentiation and decreased proliferation. In mammalian cells there are three isoforms of the C-subunit and the two major isoforms (C α and C β) have multiple splice variants that introduce diversity into the first exon (Skalhegg and Tasken, 2000). This isoform diversity is an important mechanism for achieving specificity in PKA signaling. All the C subunits (C α , C β , C γ) have catalytic

core motifs that are common to all protein kinases. The catalytic subunit is a nearly globular protein, of approximately 250 amino acids, with two lobes: a small and highly dynamic amino-terminal lobe (N-lobe) that is mostly beta strands, involved in MgATP-binding, and a larger mostly helical carboxy-terminal lobe (C-lobe) that contains much of the catalytic machinery as well as the major substrate docking sites. Both MgATP and the peptide come together for catalysis in the cleft between the two lobes. In addition to the core, the C-subunit of PKA is flanked by an N-terminal tail (N-tail) and a C-terminal tail (C-tail). These tails are an integral part of the C-subunit. Both are anchored to the N- and C-lobes of the core and thus can be thought of as cis-regulatory elements (Kim et al., 2007; Wu et al., 2007). The C-tail (residues 301–350), in particular, is an integral part of the active enzyme. The crystal structure of the murine $C\alpha$ subunit was the first protein kinase crystal structure available and has served as a template for modeling of all the other kinases (Knighton et al., 1991a).

PKA signaling and cancer

Several converging data reveal that the cAMP/PKA signaling pathway is altered in different cancers and may be exploited for cancer therapy and/or diagnosis (Naviglio et al., 2009a).

Cell cycle regulation is a key event in cancer development. Multiple intracellular signaling pathways modulate various events during cell cycle progression. cAMP and PKA play different roles in this process (Stork and Schmitt, 2002). Low cAMP levels are detected at mitosis, while higher levels are present in G1 and early S; on the other hand, PKA phosphorylates macromolecular complexes responsible for the destruction of mitotic cyclins and separation of the sister chromatids at anaphase-metaphase transition (Ferrari, 2006). PKA may act synergistically with Epac to induce mitogenesis in endocrine cells (Hochbaum et al., 2008).

By modulating the timing and localization of cAMP production, it is possible to

affect the activation of PKA (and also of other cAMP effectors), that in turn can act on the RAS/ERK and/or other signaling pathways, involved in cell cycle progression (Waschek et al., 2006; Stork and Schmitt, 2002).

The cAMP/PKA pathway has been reported to stimulate cell growth in many cell types while inhibiting it in others (Stork and Schmitt, 2002; Insel et al., 2012). An involvement of PKA in neoplastic transformation and tumor growth, especially in the onset and maintenance of endocrine tumors (hormone-responsive tissues), mainly of the corticotroph axis (pituitary and adrenal cortex) and the thyroid, is clearly indicated (Rivas and Santisteban, 2003; Mantovani et al., 2008a).

Moreover, the $RI\alpha$ expression, both at protein and mRNA level, has been found to be up-regulated in a series of transformed cell lines and human tumors (Bradbury et al., 1994; McDaid et al., 1999; Miller, 2002; Mantovani et al., 2008b; Loilome et al., 2011). Indeed several studies have indicated that inhibition of $RI\alpha$ expression through antisense oligonucleotides resulted in growth arrest of several tumor cell lines (Cho-Chung, 2004). On the other hand, overexpression of $RII\beta$ inhibits cancer cell growth and induces a reverted phenotype in various cancer cell lines (Nesterova et al., 1996), including prostate carcinoma cells. In prostate tumors, the cAMP pathway may also interact with the androgen receptor, by enhancing its activation (Merkle and Hoffmann, 2011).

Thus, uncontrolled proliferation and malignant transformation have been associated mainly with an increase of RI expression or changes in the ratio of $PKA-I$ and $-II$ (Bossis and Stratakis, 2004). Accordingly, the synthesis of RI and RII subunits and the relative abundance of $PKA-I$ and $PKA-II$ isoforms are differentially regulated during differentiation, cell growth, and neoplastic transformation, with expression of $PKA-II$ predominantly found in normal non-proliferating tissues and in growth-arrested cells, whereas enhanced levels of $PKA-I$ are detected stead-

ily in tumor cells and transiently in normal cells exposed to mitogenic stimuli (Neary et al., 2004). In addition, PKA-I and its regulatory subunit RI α are induced following transformation by certain oncogenes, such as ras (Neary et al., 2004).

On the other hand, RI α -inactivating mutations (germline or somatic mutations) result in a higher PKA activity by enzymatic studies, are associated with altered PKA subunits expression and aberrant PKA signaling, and have been found to cause primary pigmented nodular adrenocortical disease, the Carney complex, a multiple neoplasia syndrome, and sporadic endocrine tumors (Kirschner et al., 2000; Bossis et al., 2004; Bourdeau et al., 2006). Moreover, RI α null mouse embryonic fibroblasts (MEFs), showing a constitutive PKA activation, became immortalized in correlation with up-regulation of D-type cyclins (Nadella and Kirschner, 2005) and showed a decreased autophagy, a mechanism that can be associated with transformation inhibition (Mavrakis et al., 2006; Sharma et al., 2014).

Overall, overexpression of PKA-I isoform, as compared with the PKA-II one, is considered a hallmark of most human tumors, correlating with more serious clinicopathological features in several tumor types (Cho-Chung and Nesterova, 2005; Tortora and Ciardiello, 2002). Furthermore, PKA catalytic β subunit has been shown to be a direct transcriptional target of c-MYC, and proposed as a crucial component of the program by which constitutive c-MYC expression contributes to cell transformation (Wu et al., 2002).

The PKA pathway has become of great interest to the study of aging, since mutations that cause a reduction in PKA signaling have been shown to extend lifespan in yeast, and to both delay the incidence and severity of age-related disease, and to promote leanness and longevity in mice. There is increasing interest in the potential for the inhibition or redistribution of adiposity to attenuate aging and obesity-related diseases, including cancer, since obesity is associated with impaired

function of most organ systems, and is a strong risk factor for both shortened life span and tumors. Therefore, gene signaling pathways such as PKA, that play a key role in the regulation of metabolism and triglyceride storage, are potential inhibitory targets for obesity and aging intervention (Enns and Ladiges, 2010).

At this regard, it has been found that PKA catalytic β subunit is involved in diet-induced obesity, since C β subunit null animals appear overtly normal when fed standard rodent chow, whereas they are protected from diet-induced obesity, steatosis, dyslipoproteinemia and insulin resistance, without any differences in caloric intake or locomotor activity (Enns et al., 2009).

Notably, given the relevant role of obesity-linked cytokine leptin in breast cancer growth and metastasis, the leptin system has emerged as a new and promising therapeutic target for breast cancer and strategies to counteract biological effects of this obesity-linked cytokine are warranted (Gertler and Solomon, 2013). Interestingly, our previous studies provided initial evidence for the efficacy of cAMP elevation against the oncogenic effects of leptin in triple negative breast cancer cells, via PKA modulation (Spina et al., 2013).

Recently, we demonstrated that in MDA-MB-231 breast cancer cells, intracellular cAMP elevation completely abrogates both ERK1/2 and STAT3 phosphorylation in response to leptin, strongly lowers protein levels of both regulatory RI α and catalytic subunits of PKA, with a consistent reduction of CREB phosphorylation, and inhibits both leptin-induced proliferation and migration. (Naviglio et al., 2009b; Naviglio et al., 2010; Spina et al., 2012).

Another function, in which PKA may operate and may be dysregulated in cancer, is the actin-based cell migration, that involves cytoskeleton remodeling. PKA regulates actin dynamics, by targeting structural proteins, like actin, integrins, VASP and myosin light chain, and regulatory proteins, like Rho GTPases, Src kinases, p21-

activated kinases, phosphatases and proteases (Howe, 2004). The involvement of PKA in migration of breast carcinoma cells has been described (Jiang et al., 2009) and also in ovarian cancer cell migration and invasion (McKenzie et al., 2011).

Recently, it has also been shown that hypoxia enhances PKA activity by up-regulating PKA gene expression in a HIF dependent mechanism and that PKA plays a key role in hypoxia-mediated EMT, migration, and invasion in lung cancer cells (Shaikh et al., 2012).

Furthermore, PKA has been clearly shown to be involved in drug resistance in human cancer cells (Gausdal et al., 2013; de Leeuw et al., 2013).

Additionally, recent clinical studies, either measuring autoantibodies for PKA (Nesterova et al., 2006) or its enzymatic activity (Wang et al., 2007) in serum patients, strongly suggest that PKA may function as a cancer marker for various human cancers and can be used in cancer detection and for monitoring response to therapy.

Overall, based on the above considerations, PKA selective targeting in antitumour strategies has become very attractive and will be discussed below.

TARGETING PKA IN CANCER THERAPY

Site-selective cAMP analogs and antisense strategy

The interest in cyclic nucleotides as therapeutics against cancer started in the late 1980s, when Cho-Chung and colleagues discovered that 8-Cl-cAMP, a potent site-selective analog of cAMP, induced growth inhibition *in vitro* and *in vivo* in a broad spectrum of human carcinomas (breast, colon, lung), fibrosarcomas, and leukemias at micromolar concentrations, and in animal models. Since then other cAMP analogs have been developed, but further studies have been mainly conducted with 8-Cl-cAMP, most readily available by synthesis and promising enough to be tested as a drug (Schwede et al., 2000).

8-Cl-cAMP can discriminate between the two cAMP binding sites (sites A and B) on the R-subunits (RI and RII) of PKA-I and -II. 8-Cl-cAMP binds with similar high affinity to both sites A and B of RI. In contrast, it binds with high affinity to site B of RII, but with low affinity to site A, which may keep this isozyme in its nonactivated holoenzyme form (Schwede et al., 2000). As above focused, overexpression of PKA-I isoform occurs in most human tumors and has been associated with cell transformation and proliferation while growth arrest and differentiation have been linked to a decreased ratio of RI/RII. 8-Cl-cAMP was found to modulate RI and RII levels, leading to the restoration of a more natural RI/RII balance in cancer cells (Rohlf et al., 1993; Noguchi et al., 1998). 8-Cl-cAMP is able to down-regulate RI α , perhaps by facilitating the degradation of the protein after its dissociation from the PKA holoenzyme, while RII β expression is up-regulated at the transcriptional level or not affected, both leading to an increased RII/RI intracellular ratio. In preclinical studies, 8-Cl-cAMP was shown to inhibit the expression of c-myc and c-ras, to revert the transformed phenotype, and to cause inhibition of cancer cell growth through both anti-proliferation and pro-apoptotic mechanisms (Cho-Chung and Nesterova, 2005).

Yet, despite the well-documented effects of 8-Cl-cAMP and the above pioneering studies by the group of Yoon Cho-Chung, actually there is no common agreement on its mechanism of action, and the results of more recent studies suggest that the effects of 8-Cl-cAMP might be also mediated by its metabolite 8-Cl-adenosine and might be independent of PKA activation and/or alterations of the ratio between type I and type II R subunits (Robinson-White et al., 2008; Lucchi et al., 2011; Choi et al., 2013).

However, although the mechanism of action of 8-Cl-cAMP is debated and it is still not completely clear whether 8-Cl-cAMP acts as a pharmacophore itself or, at least in part, as a prodrug for 8-Cl-adenosine via a PKA-independent manner, recently, it has been

evaluated in phase I/II clinical trials as an anticancer agent (Propper et al., 1999; Tortora and Ciardiello, 2002).

Clear evidence that RI subunit of PKA was a positive effector of cancer cell growth was provided by antisense strategy data (Cho-Chung, 2004). A synthetic RI antisense oligodeoxynucleotide (ODN) corresponding to the N-terminus (the first 21 bases) of the human RI α produced growth inhibition in human cancer cells of epithelial origin, including breast (MCF-7), colon (LS-174T), and gastric (TMK-1) carcinoma and neuroblastoma (SK-N-SH) cells, as well as HL-60 leukemia cells with no sign of cytotoxicity. Moreover, treatment with RI α antisense phosphorothioate oligodeoxynucleotide (PS-ODN) brought about a marked reduction in RI α protein levels accompanied by an increase in RII β protein levels due to an increase in RII β protein half-life. This compensatory stabilization of RII protein represents an important biochemical mechanism of RI antisense that ensures both a depletion of growth-promoting RI subunit and an increase of RII, the growth-inhibitory and differentiation-inducing protein, leading to sustained inhibition of tumor cell growth (Nesterova et al., 2000). Importantly, a single subcutaneous injection of RI α antisense PS-ODN to 8-13 codons of human RI into nude mice bearing LS-174T human colon carcinoma resulted both in a reduced RI α expression and an almost complete suppression of tumor growth for up to 14 days of examination without apparent sign of systemic toxicity, whereas tumors in untreated, saline-treated, or control antisense-treated animals showed continued growth (Nesterova and Cho-Chung, 1995). Furthermore, a second-generation RNA-DNA mixed-backbone (MBO) RI α antisense ODN containing both phosphorothioate-modified nucleosides and ribonucleosides, more stable and resistant to nucleases, has been shown to cause growth arrest and differentiation in a variety of cancer cell lines in vitro and to exhibit antitumor effects in animal models of breast, colon, prostate and lung cancer (Cho-

Chung et al., 1999; Hensley et al., 2011). The RNA-DNA mixed-backbone (MBO) RI α antisense has demonstrated increased biologic activity, minimal polyanionic or immunostimulatory side effects, improved in vivo stability, and, remarkably, synergizing effects with several class of cytotoxic drugs, oral efficacy. (Wang et al., 1999, 2002).

Notably, the MBO AS-PKA-I (defined GEM 231) has recently been used for clinical studies (Mani et al., 2003; Goel et al., 2003, 2006; Tortora and Ciardiello, 2003).

Novel perspectives by new targeted agents

As the second largest group after G-protein-coupled receptors, there are more than 500 protein kinases. Because of their critical effects on cell function, their activity is tightly regulated, and thus abnormal phosphorylation is linked to various diseases, including cancer.

Accordingly, protein kinases have become very attractive drug targets (Arencibia et al., 2013). Thus, designing novel kinase specific inhibitors is a major international effort.

To date, a majority of protein kinase inhibitors with clinical applications have developed toward the ATP-binding site (Han et al., 2012).

Although many ATP-competitive small molecule inhibitors have demonstrated their potency, they have a limitation in their selectivity because of the highly conserved region of the ATP-binding site and also because these inhibitors have to compete with high concentration of intracellular ATP.

On the other hand, substrate recognition by protein kinases exhibits remarkable specificity, despite their structural and sequence homologies in the catalytic domains (Pinna and Ruzzene, 1996).

Because the substrate-binding domain is much more diverse than the ATP-binding site, substrate-competitive inhibitors are expected to show higher selectivity (Han et al., 2012).

Heat-stable protein kinase A inhibitor (PKI) purified and characterized in the early

1970s became the first head in the development of peptide inhibitors of protein kinases. PKI interacts specifically with the catalytic domain of PKA, thereby inhibiting kinase activity with a K_i of 0.2 nM in the presence of ATP (Cheng et al., 1986).

PKI (5-24) is a potent, competitive, synthetic peptide inhibitor of PKA derived from the active domain of the naturally-occurring heat-stable inhibitor protein PKI. This pseudosubstrate inhibitor peptide mimics the protein substrate by binding to the catalytic site via the arginine-cluster basic subsite, which provides high specificity. PKA catalytic subunit residues Tyr235 and Phe239 form a sandwich-like structure with residue Phe10 of PKI (5-24); this is a prominent enzyme-substrate interaction site (Knighton et al., 1991b).

Despite their selectivity, clinical applications of such substrate-competitive inhibitors are frequently hampered by several obstacles including permeability into cells, susceptibility to proteases and potential immunogenicity. Therefore, more efforts have been directed to the discovery and development of substrate competitive inhibitors, particularly considering the clinical applicability.

Generally, the design of substrate-competitive inhibitors requires comprehensive understanding of structural interaction of protein kinases with substrates or regulator proteins.

PKA was one of the first protein kinases to be discovered, the first to be sequenced and then cloned and the elucidation of its structure provided the first three dimensional template for this family (Knighton et al., 1991a; Madhusudan et al., 2002). Moreover, the structures of the regulatory subunits of PKA also have been elucidated previously (Su et al., 1995; Diller et al., 2001), but it is only recently that the structure solution of holoenzyme complexes have been resolved (Kim et al., 2007; Wu et al., 2007). From these structures the kinase has started to be considered as a scaffold, in addition to its role as a catalyst; in fact every part of its surface seems committed to some type of pro-

tein:protein interaction and these interactions appear to be as essential to its function as is phosphoryl transfer. The regulatory and catalytic subunits have been considered as separate proteins for more than a decade where they served as prototypes for the protein kinase superfamily and for cAMP binding domains, respectively. Now, instead, they can be considered as part of larger protein complexes and the understanding of how regulatory and catalytic subunits contribute to the assembly and disassembly of macromolecular signaling complexes will be made a great deal easier by these structures.

In other words, by solving crystal structures of holoenzyme complexes of PKA, the molecular features required for inhibition and for cAMP-induced activation and the entire range of strategies for designing inhibitors and interfering with PKA signaling can be fully appreciated (Taylor et al., 2013).

Thus, the approach to PKA inhibitor design could be not aimed exclusively at molecules that target the ATP binding pocket and substrate tethering sites for the catalytic subunit, but also inhibitors that target the activation of the kinase could be designed and are presumed very attractive as therapeutic agents.

The regulatory subunits, for instance, undergo major conformational changes as they release cAMP and wrap around the catalytic subunit. In the process of binding to the catalytic subunit, the cAMP binding sites (CBDs) are completely restructured. The Phosphate Binding Cassette (PBC) where the ribose phosphate docks, for example, is far removed from the residues that cap the adenine ring in the holoenzyme complex. This provides a new paradigm for designing novel agonists or antagonists for PKA (Taylor et al., 2013).

The AKAPs introduce another level of complexity into PKA signaling by localizing PKA in close proximity to its physiological substrates. A strategy aimed at disrupting PKA targeting to substrates is predicted to be equally effective. The docking motifs are also valid targets for designing inhibitors that

disrupt targeting. Furthermore, with the structure solutions of targeting motifs, novel mechanisms for disrupting targeting are also being implemented (Tröger et al., 2012).

Moreover, an additional strategy for targeting PKA activity is to affect cAMP levels by manipulating its synthesis and/or degradation, via adenylyl cyclases and/or subtype-specific phosphodiesterase, respectively (Pavan et al., 2009; Maurice et al., 2014).

CONCLUDING REMARKS

The protein kinases play a key regulatory role in cellular signaling pathways and their abnormal phosphorylation activity is inseparably linked with various human diseases, including cancer.

Accordingly, protein kinases have become invaluable drug targets and considerable effort has gone into the discovery of protein kinase inhibitors.

PKA has also emerged as major therapeutic target. PKA targeting is largely known to control cell growth in many cancer types in vitro and in vivo; remarkably, targeting PKA by either site-selective cAMP analogs or antisense approaches has clearly shown antitumor activity in cancer patients. However, discovering further PKA inhibitors is desirable.

Although strategies including bioinformatics, computational modeling, and high-throughput screening are often employed for designing specific kinase inhibitors, an invaluable guidance in developing inhibitors and interfering with PKA signaling will certainly rise from the structure solution of PKA holoenzyme complexes.

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