

Identification of cGMP-Kinase Complexes by Affinity Chromatography

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1. Introduction

During the last two decades it emerged that cGMP-dependent protein kinases (PKG) act as signalling molecules with pleiotropic physiological functions e.g. in the cardiovascular system, in the gastrointestinal tract, immune system and central nervous system (Hofmann et al., 2006). The cellular effects of PKG are transmitted by its phosphorylated substrates. The identification of different PKG substrates in the diverse organs and cells and the analysis of the interaction of PKG with its substrates are important issues which are strongly targeted by several research groups recently (summarized in (Schlossmann & Desch, 2009)). An important step for elucidating the identity and function of cGMP-kinase substrate proteins was the use of affinity chromatography methods utilizing immunoprecipitation or cyclic nucleotide based affinity columns. Thereby, a ternary complex of cGMP-kinase was purified which consists of the PKG1 isoform PKG1 β , the inositol-trisphosphate receptor type 1 (InsP₃R1) and the inositol-trisphosphate receptor-associated cGMP-kinase substrate protein (IRAG). Functional analysis by genetic deletion of IRAG in mice revealed that IRAG is essential for relaxation of vascular smooth muscle and for inhibition of platelet aggregation. Meanwhile, it was elucidated that IRAG also interacts with further isoforms of InsP₃R, namely InsP₃R2 and InsP₃R3. Furthermore, the ternary PKG1 core complex associates with several other proteins which might tune the cellular function of PKG1 in various tissues. In comparison, the association of PKG1 α with substrate proteins, MYPT1 or RGS2, was also identified by affinity chromatography including immunoprecipitation, GST-fusion proteins and/or His-tagged proteins. Therefore, the stable interaction of PKG with its substrate proteins might be a common theme and might lead to the view that direct interactions convert the intracellular function of PKG in analogy to the mechanisms which were identified for cAMP-dependent protein kinases. Therefore, affinity chromatography methods are essential tools for the identification of PKG substrate proteins and function in cells and tissues. The present review will give an overview of the structural and functional features of PKGs and of diverse PKG complexes which were identified by different affinity chromatographic techniques.

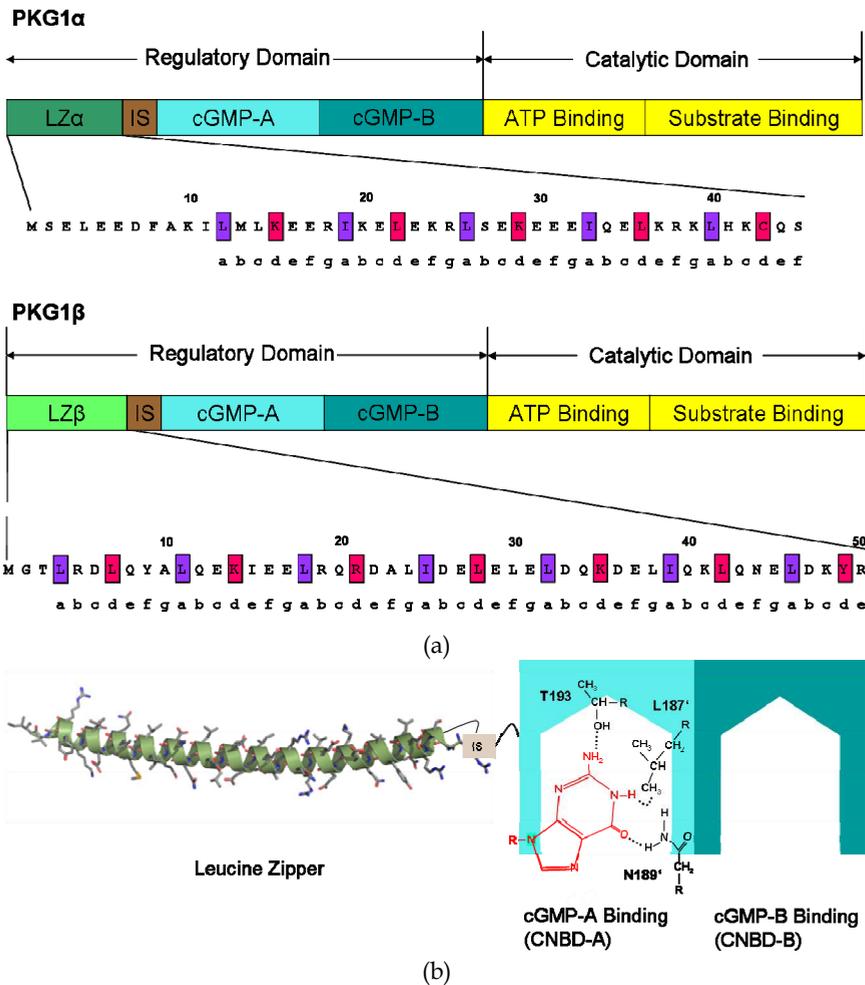
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2. cGMP-dependent protein kinases (PKG): Structure and function

PKG are stimulated by cGMP which is a key cellular second messenger. cGMP is synthesized by cytosolic soluble guanylyl cyclases (sGC) and membrane-bound particulate guanylyl cyclases (pGC). sGC are activated by the gaseous molecule nitric oxide (NO). pGC are integral membrane proteins which are stimulated by the natriuretic peptides ANP, BNP, CNP or guanylin/uroguanylin. The different intracellular locations of these enzymes lead to the view that the localized synthesis of cGMP could differentiate the alternate function of NO and natriuretic peptides (Castro et al., 2010)

PKGs are Ser/Thr kinases which phosphorylate its substrates at the recognition site K/R K/R X S/T (one letter code of aa; X can be each aa). PKGs are expressed as three different proteins: PKG1 α with 676 aa, PKG1 β with 686 aa, PKG2 with 762 aa. PKG1 and PKG2 are encoded by two different genes, *prkg1* and *prkg2*. The PKG1 isoforms are cytosolic enzymes, whereas PKG2 is plasma membrane-bound by an N-terminal myristoylation modification. The two PKG1 isoforms PKG1 α and PKG1 β are expressed from the *prkg1* gene by differential splicing and thereby differ in the N-terminal first ~100 aa which reside isoleucine/leucine zipper regions (LZ) (Fig. 1). These LZ exhibit several isoform-specific functions: homodimerization, recognition of specific-substrates, intracellular targeting. Furthermore, the K_a for cGMP-activation of PKG1 isoforms varies more than 10-fold. The K_a (cGMP) of PKG1 α is 0.059 μ M and of PKG1 β is 0.79 μ M. Recently, the structural features of PKG1 could be more specified by the first crystal structures of the N-terminal leucine zipper of PKG1 β and the cGMP-binding domains of PKG1 β (Casteel et al., 2011; Kim et al., 2011). Homodimerization of PKG1 β is mediated by eight Ile/Leu heptad repeats. Thereby, hydrophilic aa residues are coordinated in a "knobs into holes" structure which assists the dimer formation. NMR studies of the PKG1 α N-terminal domain (aa 1-39) revealed an α -helical structure with a parallel monomeric association of the coiled-coil domain which warrants PKG1 α homodimerization through fixed electrostatic interactions. The inhibitory sequence (IS) contains the substrate-like sequence PRT⁵⁹TR with autophosphorylation site (Thr⁵⁹) of PKG1 α (Aitken et al., 1984) and the presumed pseudosubstrate KRQAISAE of PKG1 β (Kemp & Pearson, 1990; Ruth et al., 1997). The IS inactivates the kinase when cGMP is absent.

Upon cGMP binding the kinase is activated. The holo-enzymes of PKG1 α or PKG1 β contain two different cyclic nucleotide-binding domains CNBD-A and CNBD-B (Fig. 1). The recently obtained cocrystals of PKG1 β (aa92-227) containing the N-terminal cyclic nucleotide binding domain of PKG1 β (CNBD-A) together with cGMP and cAMP revealed new insights into the cyclic nucleotide-binding mechanism (Kim et al., 2011). cGMP attaches only in the *syn* conformation to CNBD-A, whereas cAMP in either *syn* or *anti* configuration. Surprisingly, CNBD-A binds both cGMP and cAMP with high affinity. Only a 2-fold preference was found for cGMP. Therefore, a possible cooperative action of CNBD-A and CNBD-B in cyclic-nucleotide-binding is proposed which should ensure a more differential cyclic nucleotide binding profile of PKG1. The recently published crystal structure of the regulatory domain containing both cGMP-binding sites of PKG1 α (aa78-355) revealed a switch helix which promotes the formation of a hydrophobic interface between both cGMP-binding sites (Osborne et al., 2011). The regulatory domain is followed by the catalytic domain comprising an ATP binding - and a substrate binding domain. The 3-dimensional structure of these domains was not reported so far. Therefore, the structural elucidation of these different PKG1-domains and the holo-enzyme are still new challenges which will be essential for the functional insight of PKG1.



(A) The PKG1 is subdivided into the regulatory domain at the N terminus and the catalytic domain at the C terminus. The regulatory domain consists of the leucine zipper (LZ), the inhibitory sequence (IS) and the two cGMP binding pockets (cGMP-A/B = CNBD-A/B). The LZ is necessary for dimerization and docking to the substrates (Casteel et al., 2011). The primary sequences of the N-terminal 45 amino acids of the PKG1 α LZ and of the N-terminal 50 amino acids of the PKG1 β LZ are illustrated. The amino acids are grouped in a heptad repeat (a-g). The a-positions exhibit primary leucine or isoleucine residues (shaded in lilac) and the d-positions are mostly charged or hydrophilic residues (shaded in pink). (B) Scheme of PKG1 β regulatory domain structure (Casteel et al., 2011). Leucine Zipper: The α -helical structure is depicted in green. Side chains in the helix are marked. The linker sequence between the leucine zipper and the autoinhibitory domain is variable between PKG1 α and PKG1 β , but is not important for the PKG1 α or PKG1 β phenotype (Ruth et al., 1997). IS: The inhibitory sequence is schematically drawn. It determines the high affinity PKG1 α and the low affinity PKG1 β phenotype (Ruth et al., 1997). cGMP-A binding pocket: cGMP binds in a *syn* conformation to CNBD-A (see 3.). The essential amino acids (T193, L187, N189) for cGMP-binding are shown.

Fig. 1. Structural requirements for interaction of PKG1 α and PKG1 β with their substrates

PKG1α			
Substrate	MW(kDa)	Method for identification of interaction	Substrate function
MYPT1	130	Co-immunoprecipitation Fusion proteins	Enhanced myosin phosphatase activity mediating calcium desensitization
RGS2	24	Co-immunoprecipitation Fusion proteins	Inhibition of G α_q mediated IP $_3$ synthesis
Reference (Surks et al., 1999), (Woodbridge et al., 2004) (Sun et al., 2005), (Tang et al., 2003)			
PKG1β			
Substrate	MW(kDa)	Method for identification of interaction	Substrate function
IRAG- InsP $_3$ R1/ InsP $_3$ R2/ InsP $_3$ R3	125 230	Co-immunoprecipitation Fusion proteins cGMP-agarose	Inhibition of calcium release from IP $_3$ sensitive stores after phosphorylation of IRAG Smooth muscle relaxation Inhibition of platelet activation
PDE5	100	Co-immunoprecipitation cGMP-agarose	Enhanced cGMP degradation
Phospho-lamban	6	Co-immunoprecipitation cGMP-agarose	Increase of calcium uptake by SERCA (sarcolemmal reticulum Ca $^{2+}$ -ATPase)
TFII-I	120-150	Fusion proteins	Coordinating the activity of multiple transcription factors, signaling molecules and histone deacetylases
Reference (Hang et al., 1999), (Casteel et al., 2005), (Schlossmann et al., 2000), (Geisshoringer et al., 2004), (Desch et al., 2010), (Schinner et al., 2011), (Masuda et al., 2010), (Rybalkin et al., 2002), (Wilson et al., 2008), (Koller et al., 2003), (Lalli et al., 1999) (Casteel et al., 2005), (Casteel et al., 2002) (Roy, 2001), (Wen et al., 2003)			
PKG1			
Substrate	MW(kDa)	Method for identification of interaction	Substrate function
CRP4	22.5	Fusion proteins	Noctive behaviour in spinal cord Enhanced transcriptional activation of smooth muscle specific proteins; complex with the serum response factor (SRF)
Reference (Schmidtko et al., 2008) (Huber et al., 2000), (Zhang et al., 2007)			

Table 1. Identification of PKG1 interaction with substrates.

The table gives an overview of substrates interacting with PKG1 α , PKG1 β or both PKG1 isoforms, their molecular weights, the methods for identification of interaction and their main function.

PKG1 exhibits various functions e.g. in the cardiovascular system, in the lung, in the intestine, in platelets and in the central nervous system. PKG1 is important for the relaxation of vascular and gastrointestinal smooth muscle. This enzyme inhibits platelet activation and thereby protects against arterial thrombosis. Furthermore, in the central nervous system PKG1 is involved in processes of learning and memory (Hofmann et al., 2009). In some tissues - e.g. smooth muscles - both PKG1 isoforms are expressed. However, there are tissues where only the α -isoform is present e.g. in the lung or in the cerebellum, whereas in other cells exclusively the β -isoform is found e.g. in the human platelets or in the hippocampus. These different locations lead to the view that both isoforms exhibit different functions in tissues and cells. Therefore, the identification of isoform-specific substrates was a breakthrough to dissect the individual functions of the PKG1 α and PKG1 β . Particularly, regarding smooth muscle contractility the identification of substrates of PKG1 α (regulator of G-protein signalling 2 (RGS2) and myosin phosphatase subunit 1 (MYPT1)) and of PKG1 β (inositol trisphosphate receptor-associated cGMP kinase substrate protein (IRAG)) substantiated a detailed view of functional regulation by these PKG1 isoforms. RGS2 phosphorylation regulates the intracellular activity of G_{q/11} and thereby reduces the intracellular synthesis of InsP₃ by phospholipase C. MYPT1 phosphorylation leads to calcium desensitization of the cytoskeletal contractility (Schlossmann & Desch, 2009). Phosphorylation of IRAG inhibits the intracellular calcium release via InsP₃R1. Furthermore, IRAG is an important substrate for the NO/cGMP-dependent inhibition of platelet function and thereby prevents arterial thrombosis (Hofmann et al., 2006; Schlossmann & Desch, 2011). Various other substrates were identified in tissues and cells which lead to more precise understanding of physiological PKG functions (Hofmann et al., 2009; Schlossmann & Desch, 2009). Particularly, the identification of different PKG1-complexes together with its substrates directed to specific signalling pathways of the PKG1 isoforms. The elucidation of these complexes by affinity chromatography will be reviewed below in part 3 (PKG complexes by affinity chromatography).

3. PKG-complexes by affinity chromatography

3.1 Immunoprecipitation methods

Immunoprecipitation utilizes binding of a protein from a tissue or cell lysate to the respective primary protein-specific antibody which is linked to a Sepharose matrix followed by precipitation of the protein-antibody complex e.g. by centrifugation. If there are interacting proteins that build a stable complex with the antibody-bound protein, they can also be detected in the precipitate and hence are so called co-immunoprecipitated. The matrices used most commonly for co-immunoprecipitation experiments are protein A-Sepharose or protein G-Sepharose. Protein A and protein G are cell surface proteins from staphylococcus aureus or from streptococcus, respectively, which are able to efficiently bind immunoglobulins. Protein G-Sepharose binds goat, sheep and mouse primary antibodies with higher affinity than protein A-Sepharose (Kaboord & Perr, 2008).

For the PKG1, there are different protein complexes, which were identified by co-immunoprecipitation techniques. An identified PKG complex is the trimeric macrocomplex consisting of the PKG1 β , IRAG and the InsP₃R1 (Fig. 2, Table 1). The previously unknown protein IRAG was detected as a PKG1 substrate that could be phosphorylated in the presence of cGMP in bovine tracheal smooth muscle membranes. The multimeric complex was identified by co-immunoprecipitation experiments with the specific antibodies against the three proteins (Schlossmann et al., 2000). In each co-immunoprecipitation test, all three components of the complex could be detected in the precipitate. Later, Masuda et al. investigated the interaction between all InsP₃R-subtypes and IRAG-GFP proteins by co-immunoprecipitation experiments with anti-GFP antibody (Masuda et al., 2010). Both the neuronal S²⁺-InsP₃R1 and the peripheral S²⁻-InsP₃R1 interacted with IRAG(GFP) after transient expression in COS7 cells. By expressing IRAG Δ E12(GFP) which lacked the coiled-coil interaction site with the InsP₃R, the InsP₃R1 could not be detected in the immunoprecipitation pellet. In contrast, the PKG1 β was precipitated with IRAG and with IRAG Δ E12. In conclusion, there was a stable interaction between PKG1 β and IRAG but not between PKG1 β and the InsP₃R1. Additionally, it was shown, that InsP₃R2 and InsP₃R3 also interact with full-length IRAG but not with IRAG Δ E12 in COS7 cells (Masuda et al., 2010).

After that, in an immunoprecipitation experiment with anti-PKG1 antibody phospholamban (PLB) was identified as an additional component of the above described signalling complex (Koller et al., 2003) (Fig. 2, Table 1). Upon co-expression of InsP₃R1, IRAG α , PKG1 β and PLB in COS7 cells, InsP₃R1 and PKG1 β could be precipitated with anti-PLB antibody. Moreover, solubilized bovine tracheal smooth muscle membranes were incubated with antibodies against IRAG, PLB and PKG1 and in each precipitate the trimeric complex could be detected. Thus it was demonstrated that PLB is part of the IRAG/PKG1 β /InsP₃R1 signalling complex.

In 2008, the PDE5 could be verified as a further component of the macrocomplex in human platelets (Wilson et al., 2008) (Fig. 2, Table 1). Incubation of platelet lysates with the antisera against PDE5, PKG and InsP₃R1 allowed co-immunoprecipitation of all three proteins. IRAG was not directly detected in the complex because a primary anti-IRAG antibody was not available. However, Wilson et al. suggested that IRAG was also a component of this PDE5 signalling complex, because after ³²P-incorporation into the anti-PKG immunoprecipitate a band corresponding to the molecular weight of IRAG was detected in the autoradiogram. Furthermore, it was shown that only the PKG-associated form of PDE5 could be phosphorylated and activated after 8-Br-cGMP treatment and also only the InsP₃R1-associated PDE5 could be selectively activated by PKG1. This was demonstrated by measurements of PDE activities in different cellular fractions which contained or were devoid of InsP₃R1.

Additionally to the trimeric complex, the interaction between MYPT1 -the myosin-binding subunit (MBS) of myosin phosphatase- and PKG1 α could be verified by immunoprecipitation experiments (Fig. 2, Table 1). Lysates from saphenous vein smooth muscle cells were incubated with anti-PKG1 α and anti-MYPT1 antibodies and in the first case MYPT1 and in the second case PKG1 α was detected in the protein A-Sepharose precipitate (Surks et al., 1999). Furthermore, in the PKG1 α -immunoprecipitate, PP1 phosphatase activity was measured and also inhibited by the phosphatase inhibitor ocadaic acid. After that, to detect possible further PKG1 α substrates in the PKG1 α -PP1M complex,

cGMP and PKG1 α were added to anti-MYPT1 immunopellets. In the presence of [γ - 32 P]ATP there was a significantly increased phosphorylation of MYPT1. In 2003, it was hypothesized that the leucine zipper domains of MYPT1 and PKG1 α mediate the interaction between the two proteins (Surks & Mendelsohn, 2003). To further investigate the relevance of the MYPT1 leucine zipper for the interaction, co-immunoprecipitation experiments were performed with chicken smooth muscle cells and chicken smooth muscle tissue (Huang et al., 2004). Aorta from embryonic day 15 expressed a leucine zipper positive MYPT1 (LZ $^{+}$) and gizzard smooth muscle tissue from day 7 expressed a LZ $^{-}$ MYPT1 isoform. Both LZ $^{+}$ and LZ $^{-}$ MYPT1 isoforms associated with PKG1 and the presence of cGMP increased the binding of the MYPT1 to PKG1 in the embryonic aorta. In contrast, in cultured smooth muscle cells, neither LZ $^{+}$ nor LZ $^{-}$ MYPT1 isoforms co-immunoprecipitated with PKG1 α . These results suggested that the binding of MYPT1 to PKG1 α is not mediated by a LZ-LZ interaction. Because PKG1 α prefers binding to RR and RK motifs (Dostmann et al., 2000) and there is an RK motif in the aa 888–928 sequence of MYPT1, the relevance of this sequence was investigated (Given et al., 2007). Mutants were generated, which lack or contain this sequence and which also lack or contain the leucine zipper. These four MYPT1 mutant proteins were added to adult chicken gizzard lysate and chicken aorta lysate and co-immunoprecipitation experiments with anti-PKG1 α antibody were performed. Only the fragments that contained the amino acids 888–928 interacted with PKG1 α and the interaction was independent of the expression of the leucine zipper (Given et al., 2007). The RK motif within the aa 888–928 sequence is located at aa 916 and 917, that were mutated to alanine (MYPT1A) or glutamic acid (MYPT1E) to investigate whether these two amino acids are important for binding of PKG1 α . It was shown that MYPT1A binds to PKG1 α whereas the interaction between MYPT1E and PKG1 α is abolished. Therefore the amino acids R 916 K 917 are important for the binding of PKG1 α and the interaction depends on the charge of the amino acid residues and not on the size. At last, it was proved that an accessory protein may be necessary for interaction, because there is a loss of the MYPT1-PKG1 α interaction if partially purified PKG1 α is used in the co-immunoprecipitation experiment with the MYPT1 fragment (Given et al., 2007).

Additionally to the IRAG- and the MYPT1- PKG1 complexes, an interaction between PKG1 α and the regulator of G-protein signalling-2 (RGS2) was demonstrated (Fig. 2, Table 1). When recombinant [35 S] RGS2 protein and recombinant PKG1 α were combined, a co-immunoprecipitation of the RGS2 protein with PKG1 α was detected (Tang et al., 2003).

3.2 Affinity chromatography by GST-fusion or His-tagged proteins

The interaction between two proteins can also be investigated by generation and analysis of GST- and His-tagged fusion proteins. A GST-tagged fusion protein contains the glutathione S-transferase enzyme and can be purified with glutathione beads. A Polyhistidine-tag consists of at least five histidine residues and His-tagged fusion proteins bind to Ni $^{2+}$ -matrices. These methods were used for the analysis of the interaction of substrate proteins with PKG1 α or PKG1 β . The interactions of the different GST- and His-tagged proteins are summarized in table 2.

The interaction between RGS2 and PKG1 α was also demonstrated by analysing GST-fusion proteins (Tang et al., 2003). A GST-PKG1 α (1–59) fusion protein, which contained the PKG1 α leucine zipper domain, interacted with the [35 S] RGS2 protein. The N-terminal region of

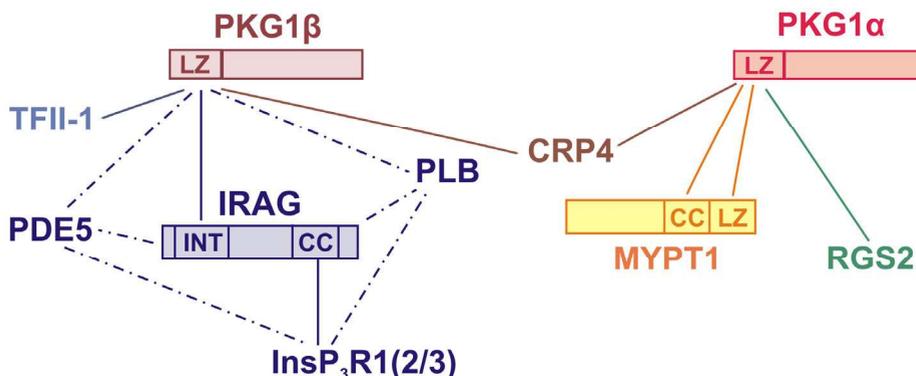


Fig. 2. PKG1 α and PKG1 β complexes identified by affinity chromatography methods. The filled lines indicate confirmed stable interactions between two complex proteins. When the respective interaction sites are known they are illustrated in the figure. The broken lines indicate putative interactions of complex proteins.

Complex proteins: PKG1 α/β : cGMP-dependent protein kinase 1 α/β ; IRAG: inositol-trisphosphate receptor-associated cGMP-kinase substrate protein; InsP₃R1/2/3: inositol 1,4,5-trisphosphate receptor subtype 1/2/3; PDE5: phosphodiesterase 5; TFII-1: general transcriptional regulator 1; PLB: phospholamban; CRP4: Cystein rich LIM protein 4; MYPT1: myosin targeting subunit of myosin phosphatase; RGS2: regulator of G-protein signalling 2

Interaction sites: LZ: leucine zipper; INT: IRAG-interaction site with PKG1 β ; CC: coiled-coil domain

RGS2 (GST-RGS2-N; residues 1-79) bound endogenous PKG1 α from venous and arterial VSMC lysates. In contrast, the GST-RGS2 fusion protein of the C-terminus (GST-RGS2-C (80-211)) did not interact with the cGMP-dependent protein kinase. Summing up, the N-terminus of RGS2 binds the N-terminal leucine zipper domain of PKG1 α in vascular smooth muscle cells (Fig. 2).

GST-fusion proteins were also generated to investigate the MYPT1-PKG1 α interaction (Surks et al., 1999). A GST-MYPT1 protein (GST MYPT1 (850-1030) WT), a GST-tagged protein of the C-terminus of the Myosin-binding subunit interacted with PKG1 α . After that, it was demonstrated with GST-fusion proteins of the N-termini of PKG1 α (GST PKG1 α (1-59)), PKG1 β (GST PKG1 β (1-92)) and PKG2 (GST PKG2 (1-272)) that only PKG1 α interacts with MYPT1 (Fig. 2). Subsequently, the relevance of the leucine zipper of MYPT1 for the interaction with PKG1 α was also investigated with GST-tagged proteins. GST-fusion proteins of MYPT1 mutants were generated in which two consecutive leucines were mutated to alanine (MYPT1 1007A/1014A mutant and MYPT1 1021A/1028A mutant). Only the wild-type GST-MYPT1 bound endogenous PKG1 α from human aortic smooth muscle cell lysate, the MYPT1 mutants did not interact with the cGMP-dependent protein kinase (Surks & Mendelsohn, 2003). The MYPT1 leucine zipper was also relevant for homodimerization because these leucine zipper mutant GST-MYPT1 (GST MYPT1 CT₁₈₁ L1021A/L1028A and GST MYPT1 CT₁₈₁ L1007A/L1014A) did not bind MYPT1 from VSMC lysate (Surks & Mendelsohn, 2003). On the other hand the PKG1 α leucine/isoleucine zipper

	fusion protein	interacting partner	interaction	reference
PKG1α/PKG1α	GST PKG1 α (1-59) WT	VSMC lysate: PKG1 α	yes	(Surks & Mendelsohn, 2003)
	GST PKG1 α (1-59) L26P	VSMC lysate: PKG1 α	yes	
	GST PKG1 α (1-59) L12A/I19A	VSMC lysate: PKG1 α	yes	
	GST PKG1 α (1-59) I33A/L40A	VSMC lysate: PKG1 α	yes	
MYPT1/PKG1α	GST MYPT1 CT ₁₈₁ (850-1030) WT	VSMC lysate: PKG1 α	yes	(Surks et al., 1999)
	GST PKG1 α (1-59)	VSMC lysate: MYPT1	yes	
	GST PKG1 β (1-92)	VSMC lysate: MYPT1	no	
	GST PKG2 (1-272)	VSMC lysate: MYPT1	no	
	GST MYPT1 CT ₁₈₁ (850-1030) WT	VSMC lysate: PKG1 α	yes	(Surks & Mendelsohn, 2003)
	GST MYPT1 CT ₁₈₁ (850-1030) L1021A/L1028A	VSMC lysate: PKG1 α	no	
	GST MYPT1 CT ₁₈₁ (850-1030) L1007A/L1014A	VSMC lysate: PKG1 α	yes	
	GST PKG1 α (1-59) WT	His MYPT1 CT ₁₀₀ (930-1030)	yes	(Sharma et al., 2008)
MYPT1/MYPT1	GST MYPT1 CT ₁₈₁ WT	VSMC lysate: MYPT1	yes	(Surks & Mendelsohn, 2003)
	GST MYPT1 CT ₁₈₁ L1021A/L1028A	VSMC lysate: MYPT1	no	
	GST MYPT1 CT ₁₈₁ L1007A/L1014A	VSMC lysate: MYPT1	no	
PKG1β/PKG1β	GST PKG1 β (1-110) E29K	PKG1 β E29K	yes	(Casteel et al., 2005)
	GST PKG1 β (1-110) D26K/E31R	PKG1 β D26K/E31R	yes	
IRAG/PKG1β	GST PKG1 α WT	Myc IRAG WT	no	(Casteel et al., 2005)
	GST PKG1 β WT	Myc IRAG WT	yes	
	GST PKG1 β (1-110) WT	His/Myc IRAG (1-415) WT	yes	
	GST PKG1 β (1-110) E29K	His/Myc IRAG (1-415) WT	no	
	GST PKG1 β (1-110) D26K/E31R	His/Myc IRAG (1-415) WT	no	
	GST IRAG (1-415) WT	purified PKG1 β	yes	
	GST IRAG (1-415) R120A/R124A	purified PKG1 β	no	
	GST IRAG (1-415) R124A/R125A	purified PKG1 β	no	
	GST PKG1 β WT	Myc IRAG R124A/R125A	no	
TFII-I/PKG1β	GST PKG1 α WT	Myc TFII-I WT	no	(Casteel et al., 2005)
	GST PKG1 β WT	Myc TFII-I WT	yes	
RGS2/PKG1α	GST RGS2-N (1-79)	VSMC lysate: PKG1 α	yes	(Tang et al., 2003)
	GST RGS2-C (80-211)	VSMC lysate: PKG1 α	no	
	GST PKG1 α (1-59) WT	[³⁵ S]RGS-2	yes	
CRP4/PKG1	Myc CRP4	PKG1 α	yes	(Zhang et al., 2007)
	Myc CRP4	PKG1 β	yes	

Table 2. Interaction of GST- and His-tagged fusion proteins (PKG1 α , PKG1 β and/or its substrate proteins). More details of these interactions are given in chapter 3.

was dispensable for formation of PKG dimers, which was shown by analysing the interaction between GST-tagged wild type and mutant amino-terminal PKG1 α and endogenous PKG1 α from VSMC lysate (Surks & Mendelsohn, 2003). Lee et al. (Lee et al., 2007) provided data that supported the importance of both the leucine zipper and the C-terminal CC domain of MYPT1 for the formation of the PKG1 α -MYPT1 complex. Therefore, the interaction between the leucine zipper of PKG1 α (PKG1 α_{1-59}) and the C-terminal CC domain of the Myosin Binding Subunit was tested (Sharma et al., 2008). A C-terminal His₆-MYPT1 CT₁₀₀ (residues 930-1030) protein which was bound to Ni²⁺-NTA resin precipitated GST-PKG1 α_{1-59} . In isothermal titration calorimetry studies and NMR experiments the importance of the residues 929-970 (MYPT1 CT₄₂) for the interaction with the leucine zipper of PKG1 α was demonstrated (Sharma et al., 2008). In short, if the leucine-zipper motif of MYPT1 is absent, the PKG1 α leucine-zipper binds to the coiled coil region and upstream segments of MYPT1 (Lee et al., 2007).

GST-fusion proteins were also generated to further investigate the interaction between IRAG and PKG1 β . A GST-tagged full-length PKG1 β interacted *in vivo* with a Myc-tagged IRAG (full-length IRAG WT) after expression of the proteins in COS7 cells (Casteel et al., 2005). Full-length PKG1 α showed no binding of IRAG. Because both PKG1 isoforms bind via salt sensitive ionic interactions, the involvement of charged residues within the leucine zipper of PKG1 β was tested. Acidic residues in the N-terminal sequence of PKG1 β were mutated and the GST-tagged mutants were expressed in E.coli. These proteins were incubated with the Myc- and His-tagged N-terminal IRAG (His/Myc IRAG (1-415) WT). Two mutants of PKG1 β (GST PKG1 β (1-110) E29K and GST PKG1 β (1-110) D26K/E31R) showed no longer binding to IRAG and therefore it was concluded that acidic residues in PKG1 β mediate the interaction with IRAG. On the other hand, basic residues in the IRAG interaction site (residues 100-132) (Ammendola et al., 2001) are necessary for binding of PKG1 β . To determine the specific amino acids, pairs of basic residues were mutated to alanine and the GST-tagged IRAG mutants were tested for their ability to bind PKG1 β *in vitro*. Some of the mutants showed completely disrupted binding (GST IRAG (1-415) R120A/R124A and GST IRAG (1-415) R124A/R125A) and others showed decreased binding to the cGMP-dependent protein kinase (Casteel et al., 2005). The E29K and D26K/E31R PKG1 β mutants, which exhibited mutations of the acidic residues that are important for the interaction with IRAG, could form stable dimers after expression in COS7 cells. Therefore, these residues are not necessary for PKG1 β homodimerization.

The importance of PKG1 homodimerization was demonstrated by the cardiovascular deficits exhibited by transgenic mice expressing a dimerization-deficient form of PKG1 α (Michael et al., 2008). To further understand the molecular details of PKG1 dimerization and the association of PKG1 dimers with GKAPs, Casteel et al. solved a crystal structure of the PKG1 β dimerization/docking domain by investigating a His-tagged PKG1 β D/D domain (Casteel et al., 2011) (described in 2.).

Similarly to the IRAG-PKG1 β interaction, the interaction between PKG1 β and the general transcriptional regulator TFII-I was investigated (Casteel et al., 2005). Like IRAG, Myc-tagged TFII-I was only bound by GST-tagged PKG1 β but not by PKG1 α . It was shown that electrostatic interactions are important for binding of PKG1 β to GST-TFII-I, because high NaCl concentrations (400 mM) disrupted binding. Acidic residues in the PKG1 β leucine zipper are

important for the interaction with basic amino acids of TFII-I which was proved by analysis of GST-tagged PKG1 β and TFII-I mutants (Casteel et al., 2005). TFII-I is not only bound but also phosphorylated by PKG1 and as a consequence of this transcriptional activation of a serum response factor-dependent reporter gene is enhanced (Casteel et al., 2002).

Fusion proteins were also generated to analyze the interaction between the cysteine-rich LIM-only protein CRP4 and PKG1 in vascular smooth muscle cells (Zhang et al., 2007). After immunoprecipitation of endogenous CRP4 from PAC1 cells PKG1 was detected in the precipitate and Myc-epitope-tagged CRP4 interacted with PKG1 α and PKG1 β after expression in CV1 cells. Additionally, it was shown that cGMP/PKG1 mediated phosphorylation of CRP4 had no effect on CRP4-PKG1 association and that Ser104 is the major PKG phosphorylation site.

3.3 Cyclic nucleotide affinity methods

As cGMP-dependent protein kinases are activated by cyclic GMP and contain cGMP binding domains they can be purified or bound by cGMP-agarose beads. The PKG1 β /IRAG/InsP₃R1 macrocomplex was not only identified by co-immunoprecipitation experiments with the respective primary antibodies but also after expression of the three proteins in COS7 cells and then incubating the cell lysate with 8-AET-cGMP-agarose beads (Ammendola et al., 2001). The cGMP-agarose affinity method was also performed with colon smooth muscle tissues from IRAG-deficient and WT mice (Desch et al., 2010). Thereby it was demonstrated that the interaction of PKG1 β and InsP₃R1 is destroyed by IRAG deletion and that the InsP₃R1 misses a stable interaction site for PKG1 β .

In 2003, Phospholamban (PLB) was identified as a component of the PKG1 β /IRAG/InsP₃R1 signalling complex after bovine tracheal microsomal membrane proteins were purified by cGMP-agarose, phosphorylated by 8-pCPT-cGMP and separated by SDS-PAGE or Tricin-SDS-PAGE (Koller et al., 2003). Beside InsP₃R1, IRAG and PKG1 β , Phospholamban and RhoA were phosphorylated in the presence of cGMP. Smooth muscle α -actin and smooth muscle calponin were also present in the cGMP-agarose complex and were identified by MALDI-TOF. Additionally, after pre-purification by cGMP-agarose and phosphorylation in presence of 8-pCPT-cGMP the microsomal membrane proteins were immunoprecipitated with anti-InsP₃R1, anti-IRAG, anti-PKG1 and anti-PLB antibody. In each case, the autoradiogram showed phosphorylated InsP₃R1, IRAG and PKG1 β which is a further indication that PLB is a component of the macrocomplex and interacts with PKG1 β .

Furthermore, PDE5 is present in the cGMP-agarose complex (Wilson et al., 2008) and an increased PDE activity was measured in the cGMP-agarose pellet after 8-Br-cGMP treatment.

Margarucci et al. investigated the rapid spatial responses of cAMP and cGMP signalling complexes induced by collagen stimulation of human platelets (Margarucci et al., 2011). The platelets were isolated from whole blood, activated by collagen related peptide (CRP) for 5 minutes and after platelet lysis the proteins were bound to a 1:1 mixture of 2-AHA-cGMP- and 8-AET-cGMP-agarose beads. After that, the proteins were eluted, digested and identified and quantified by LC-MS/MS. PKG1 β , PKG2, PDE2A and PDE5A were identified as primary and IRAG, InsP₃R1 and InsP₃R2 as secondary interactors. After CRP stimulation, the secondary interactors showed increased enrichment versus control whereas binding of PKG1 β was unaltered and the amount of PKG2 slightly decreased. Moreover, different

phosphopeptides were identified and also quantified and it was shown, that phosphorylation of IRAG at Ser670 is reduced by 60% after incubation with collagen. Additionally, the autophosphorylation of PKG1 β at Ser63 was reduced which presumably corresponded to a reduced kinase activity and lead to increased platelet activation after CRP stimulation.

4. Function of PKG complexes

4.1 IRAG-PKG1 β and the ternary PKG macrocomplex

IRAG is a 125 kDa substrate of PKG1 β which is located at the endoplasmic reticulum membrane anchored by a C-terminal tail. IRAG is expressed in high amounts in smooth muscle tissues including the vasculature, the gastrointestinal tract and the lung. Furthermore, IRAG is found in high concentration in platelets. IRAG expression is also found in lower quantity in further tissues including heart, osteoclasts and thalamus. The interaction of IRAG was identified by various methods including co-immunoprecipitation, cGMP affinity chromatography, two-hybrid analysis and mutagenesis studies. Thereby, an N-terminal located 33 aa IRAG domain (aa152-184) was identified which interacts with the Ile/Leu-zipper domain of PKG1 β but not with PKG1 α . Two arginines of the IRAG interaction domain (R124 and R125) are essential for the electrostatic interaction with acidic aa of PKG1 β (D26, E29, E31) (Casteel et al., 2005). PKG1 β and IRAG form a ternary complex together with InsP₃R1 in smooth muscle, platelets and osteoclasts. IRAG is the core component of the complex which interacts with the InsP₃R1 by its central coiled-coil domain. Deletion of this coiled-coil domain prevent the InsP₃R1 interaction but does not alter IRAG-PKG1 β binding. However, the deficiency of IRAG in IRAG-KO mice destructs the ternary complex showing that InsP₃R1 does not stably interact with PKG1 β . The function of IRAG in the ternary complex was revealed by targeted deletion of the coiled-coil domain or total deletion of IRAG in mice. Thereby, it was elucidated that IRAG is essential for NO- and ANP-mediated smooth muscle relaxation (Desch et al., 2010). IRAG deletion did not alter basal blood pressure but prevented the blood pressure drop upon LPS-induced sepsis (Desch et al., 2010). In platelets, IRAG deletion lead to hyperaggregability and an enhanced amount of platelets in the blood. The NO/cGMP-mediated inhibition of platelet activation was prevented upon IRAG deletion (Schinner et al., 2011). Concludingly, IRAG prevented arterial thrombosis (Antl et al., 2007). Recently, it was reported that IRAG is one of seven proteins which exhibited a polymorphism which was associated with enhanced platelet aggregability known to be a major factor for the incidence of cardiovascular diseases (Johnson et al., 2010). A recent study showed that IRAG might be involved in the activation and attachment of osteoclasts (Yaroslavskiy et al., 2010). Furthermore, it was reported that IRAG is also able to interact with further InsP₃R isoforms, namely InsP₃R2 and InsP₃R3 (Masuda et al., 2010). The exact mechanism of IRAG interaction with InsP₃R and the inhibition of InsP₃R gating was not established so far and remains a fundamental subject of IRAG function.

4.2 MYPT1-PKG1 α

PKG1 α interaction with MYPT1, a 130 kDa myosin-binding subunit of phosphatase 1, was revealed by co-immunoprecipitation, two hybrid analysis and mutagenesis studies. The Ile/Leu-zipper domain of PKG1 α (aa 1-59) interacts with MYPT1. There are two diverse developmentally regulated MYPT1 isoforms existing in vascular smooth muscle: an isoform containing a C-terminal leucine zipper (LZ+) which is sufficient to bind to PKG1 α and

thereby sensitizes cGMP-dependent relaxation. Nitric oxide tolerance was related to enhanced degradation of the LZ⁺-isoform. Moreover, a decreased expression of LZ⁺ was correlated with congestive heart failure. The leucine-zipper deficient isoform (LZ⁻) alternatively interacts with PKG1 α through its N1-N2 coiled-coil domain but is cGMP-insensitive (Payne et al., 2006).

4.3 RGS2-PKG1 α

The PKG1 α -Ile/Leu-Zipper domain interacts with the N-terminal 79aa of RGS2. This RGS2-domain includes an amphipathic α -helical domain with Ser46 and Ser 64. Upon cGMP-dependent phosphorylation these Ser residues enable membrane targeting. Vice versa, localization of PKG1 α at the plasma membrane was enhanced by RGS2 phosphorylation suggesting a thereby stabilized complex of these proteins. Thus, RGS2 is activated by PKG1 α and hence G_q-triggered InsP₃-synthesis is attenuated (Tang et al., 2003). Accordingly, the deletion of RGS2 in mice impairs cGMP-dependent reduction of calcium transients in vascular smooth muscle cells. Furthermore, NO-dependent reduction of blood pressure is suppressed in RGS2-deficient mice (Sun et al., 2005). Interestingly, the hypertensive phenotype was correlated to nitric oxide function only in inactive phases of mice (during the day). In contrast, sympathetic activation and increased vascular adrenergic responsiveness correlated with enhanced blood pressure of RGS2-deficient mice in the active phases (during the night) (Obst et al., 2006). Recent reports revealed that ANP-signalling is also transduced by the PKG1 α -RGS2 complex mediating the suppression of angiotensin II-induced cardiac hypertrophy (Klaiber et al., 2010).

4.4 Further PKG-substrate complexes

There are several further proteins which interact with the PKG1 isoforms. However, the elucidation of these PKG interacting proteins as PKG substrates was obtained only occasionally. The Cystein rich LIM protein CRP4 was identified as PKG1 α - and PKG1 β -interacting protein by two hybrid analysis using a smooth muscle cDNA library. CRP4 is phosphorylated as substrate by PKG1 (Huber et al., 2000). The CRP4 protein interacts via its third zinc finger domain with PKG1 associating together in a complex with serum response factor (SRF). Upon cGMP/PKG1 activation CRP4 enhances SRF/DNA association and thereby regulates the expression of smooth muscle-specific genes (Zhang et al., 2007).

A further PKG1 β interacting protein is the general transcriptional regulator TFII-1 which resides as interface a R4 helix-loop-helix motif (aa 491-628). TFII-1 interacts with basic aa to the Leu/Ile domain of PKG1 β similarly as reported for the IRAG protein. The TFII-1 interaction is cGMP-independent. TFII-1 transactivation of a serum-response element was enhanced by PKG1 β (Casteel et al., 2005).

5. Conclusion

The identification of PKG-interacting proteins by affinity chromatographic methods clarified several new signalling pathways of PKG1. Furthermore, the dissection of PKG1 isoform specific functions of PKG1 α and PKG1 β was substantiated by the identification of specific substrate proteins in several tissues e.g. in smooth muscle, in platelets and in osteoclasts as shown above. The advanced elucidation of the function of the known PKG-interacting and

substrate proteins will be a cue for the determination of pathophysiological consequences in cardiovascular, haematological and gastrointestinal diseases. Furthermore, the diverse signal transduction pathways of PKG1 isoforms and the detailed molecular analysis of their interactions and possible interferences will lead to new therapeutic horizons. However, the ubiquitous expression of PKG1 and the pleiotropic functions of the PKG1 isoforms require a very subtle intracellular signal regulation by probably a variety of further substrate proteins which were not elucidated so far. Particularly, in immunological functions, in the renal system and in the central nervous system there are a variety of mechanisms which are not explained by the current knowledge about PKG signal transduction. The use of interaction assays including affinity chromatography methods is tempting to find new pathways which will aid in the identification of new (patho)physiological aspects of PKG signalling.

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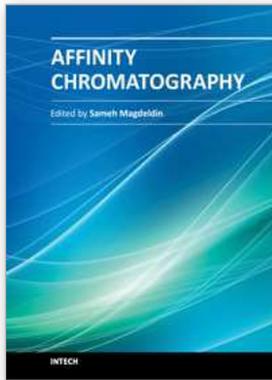
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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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