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# 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 PKG1β, the inositol-trisphosphate receptor type 1 ($InsP_3R1$) 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 $InsP_3R$, namely $InsP_3R2$ and $InsP_3R3$. 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 PKG1α 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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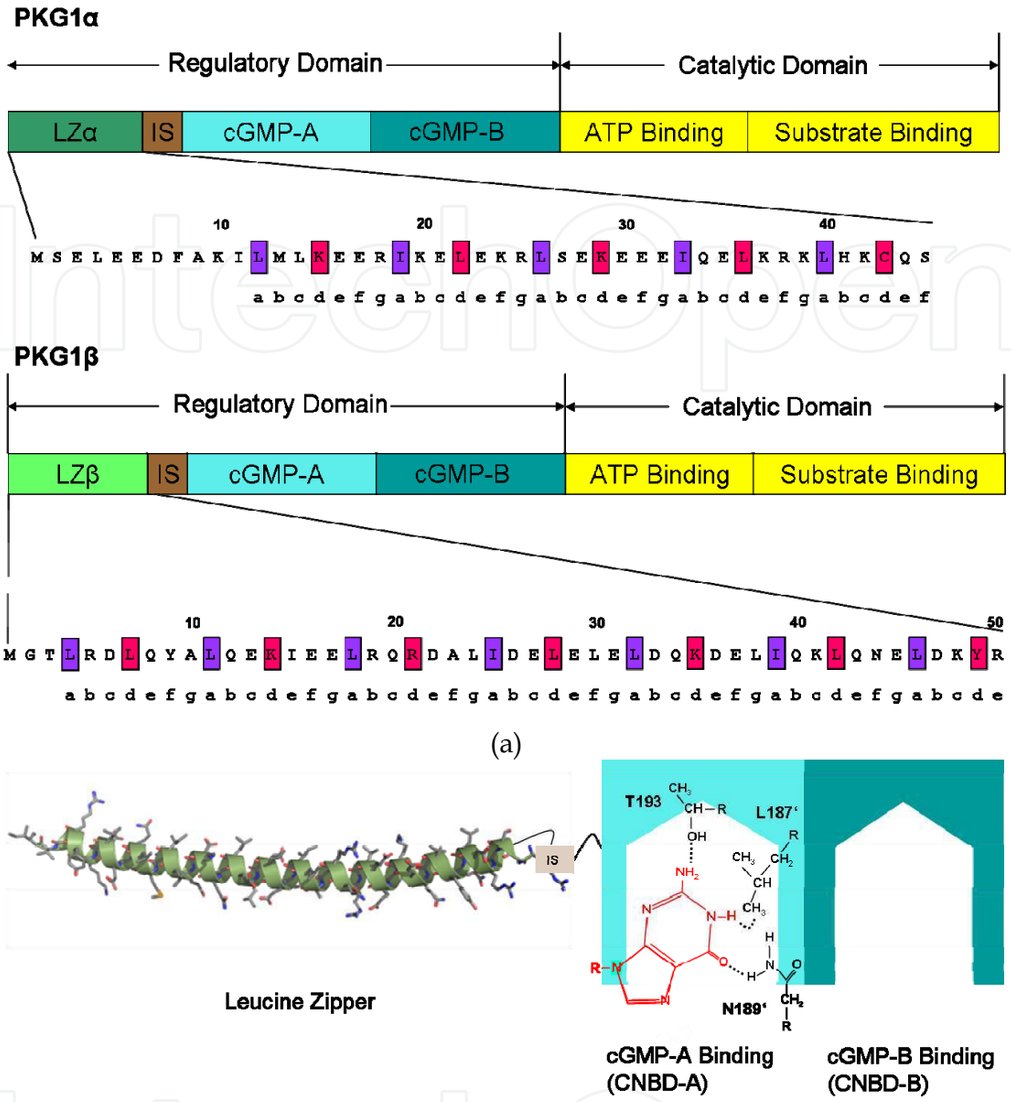
## 2. cGMP-dependent protein kinases (PKG): Structure and function

PKG are stimulated by cGMP which is a key cellular second messenger. cGMP is synthesized by cytosolic soluble guanylyl cyclases (sGC) and membrane-bound particular 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: PKG1α with 676 aa, PKG1β 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 PKG1α and PKG1β 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 PKG1α is 0.059 μM and of PKG1β is 0.79 μM. Recently, the structural features of PKG1 could be more specified by the first crystal structures of the N-terminal leucine zipper of PKG1β and the cGMP-binding domains of PKG1β (Casteel et al., 2011; Kim et al., 2011). Homodimerization of PKG1β 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 PKG1α N-terminal domain (aa 1-39) revealed an α-helical structure with a parallel monomeric association of the coiled-coil domain which warrants PKG1α homodimerization through fixed electrostatic interactions. The inhibitory sequence (IS) contains the substrate-like sequence PRT$^{59}$TR with autophosphorylation site (Thr$^{59}$) of PKG1α (Aitken et al., 1984) and the presumed pseudosubstrate KRQAISAE of PKG1β (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 PKG1α or PKG1β contain two different cyclic nucleotide-binding domains CNBD-A and CNBD-B (Fig. 1). The recently obtained cocrystals of PKG1β (aa92-227) containing the N-terminal cyclic nucleotide binding domain of PKG1β (CNBD-A) together with cGMP and cAMP revealed new insights into the cyclic nucleotide-binding mechanism (Kim et al., 2011). cGMP attachs 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 PKG1α (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 PKG1α LZ and of the N-terminal 50 amino acids of the PKG1β 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 PKG1β 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 PKG1α and PKG1β, but is not important for the PKG1α or PKG1β phenotype (Ruth et al., 1997). IS: The inhibitory sequence is schematically drawn. It determines the high affinity PKG1α and the low affinity PKG1β 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 PKG1α and PKG1β with their substrates

**PKG1α**

| Substrate | MW(kDa) | Method for identification of interaction | Substrate function | Reference |
|---|---|---|---|---|
| MYPT1 | 130 | Co-immunoprecipitation<br>Fusion proteins | Enhanced myosin phosphatase activity mediating calcium desensitization | (Surks et al., 1999), (Wooldridge et al., 2004) |
| RGS2 | 24 | Co-immunoprecipitation<br>Fusion proteins | Inhibition of $G_q$ mediated $IP_3$ synthesis | (Sun et al., 2005), (Tang et al., 2003) |

**PKG1β**

| Substrate | MW(kDa) | Method for identification of interaction | Substrate function | Reference |
|---|---|---|---|---|
| IRAG-<br>$InsP_3R1$/<br>$InsP_3R2$/<br>$InsP_3R3$ | 125<br>230 | Co-immunoprecipitation<br>Fusion proteins<br>cGMP-agarose | Inhibition of calcium release from $IP_3$ sensitive stores after phosphorylation of IRAG<br>Smooth muscle relaxation<br>Inhibition of platelet activation | (Haug et al., 1999), (Casteel et al., 2005), (Schlossmann et al., 2000), (Geiselhoringer et al., 2004), (Desch et al., 2010), (Schinner et al., 2011), (Masuda et al., 2010) |
| PDE5 | 100 | Co-immunoprecipitation<br>cGMP-agarose | Enhanced cGMP degradation | (Rybalkin et al., 2002), (Wilson et al., 2008) |
| Phospho-<br>lamban | 6 | Co-immunoprecipitation<br>cGMP-agarose | Increase of calcium uptake by SERCA (sarcoplasmic reticulum $Ca^{2+}$-ATPase) | (Koller et al., 2003), (Lalli et al., 1999) |
| TFII-1 | 120-150 | Fusion proteins | Coordinating the activity of multiple transcriptions factors, signaling molecules and histone deacetylases | (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 | Reference |
|---|---|---|---|---|
| CRP4 | 22.5 | Fusion proteins | Nociceptive behaviour in spinal cord<br>Enhanced transcriptional activation of smooth muscle specific proteins; complex with the serum response factor (SRF) | (Schmidtko et al., 2008)<br>(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 PKG1α, PKG1β 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 PKG1α and PKG1β. Particularly, regarding smooth muscle contractility the identification of substrates of PKG1α (regulator of G-protein signalling 2 (RGS2) and myosin phosphatase subunit 1 (MYPT1)) and of PKG1β (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 $InsP_3$ 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 $InsP_3R1$. 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 PKG1β, IRAG and the $InsP_3R1$ (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 $InsP_3R$-subtypes and IRAG-GFP proteins by co-immunoprecipitation experiments with anti-GFP antibody (Masuda et al., 2010). Both the neuronal $S^{2+}$-$InsP_3R1$ and the peripheral $S^{2-}$-$InsP_3R1$ interacted with IRAG(GFP) after transient expression in COS7 cells. By expressing IRAGΔE12(GFP) which lacked the coiled-coil interaction site with the $InsP_3R$, the $InsP_3R1$ could not be detected in the immunoprecipitation pellet. In contrast, the PKG1β was precipitated with IRAG and with IRAGΔE12. In conclusion, there was a stable interaction between PKG1β and IRAG but not between PKG1β and the $InsP_3R1$. Additionally, it was shown, that $InsP_3R2$ and $InsP_3R3$ also interact with full-length IRAG but not with IRAGΔE12 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 $InsP_3R1$, IRAGa, PKG1β and PLB in COS7 cells, $InsP_3R1$ and PKG1β 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/PKG1β/$InsP_3R1$ 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 $InsP_3R1$ 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 $^{32}P$-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 $InsP_3R1$-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 $InsP_3R1$.

Additionally to the trimeric complex, the interaction between MYPT1 -the myosin-binding subunit (MBS) of myosin phosphatase- and PKG1α could be verified by immunoprecipitation experiments (Fig. 2, Table 1). Lysates from saphenous vein smooth muscle cells were incubated with anti-PKG1α and anti-MYPT1 antibodies and in the first case MYPT1 and in the second case PKG1α was detected in the protein A-Sepharose precipitate (Surks et al., 1999). Furthermore, in the PKG1α-immunoprecipitate, PP1 phosphatase activity was measured and also inhibited by the phosphatase inhibitor ocadaic acid. After that, to detect possible further PKG1α substrates in the PKG1α-PP1M complex,

cGMP and PKG1α 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 PKG1α 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 PKG1α. These results suggested that the binding of MYPT1 to PKG1α is not mediated by a LZ-LZ interaction. Because PKG1α 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-PKG1α antibody were performed. Only the fragments that contained the amino acids 888-928 interacted with PKG1α 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 PKG1α. It was shown that MYPT1A binds to PKG1α whereas the interaction between MYPT1E and PKG1α is abolished. Therefore the amino acids $R^{916}$ $K^{917}$ are important for the binding of PKG1α 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-PKG1α interaction if partially purified PKG1α 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 PKG1α and the regulator of G-protein signalling-2 (RGS2) was demonstrated (Fig. 2, Table 1). When recombinant [$^{35}$S] RGS2 protein and recombinant PKG1α were combined, a co-immunoprecipitation of the RGS2 protein with PKG1α 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 PKG1α or PKG1β. The interactions of the different GST- and His-tagged proteins are summarized in table 2.

The interaction between RGS2 and PKG1α was also demonstrated by analysing GST-fusion proteins (Tang et al., 2003). A GST-PKG1α (1-59) fusion protein, which contained the PKG1α leucine zipper domain, interacted with the [$^{35}$S] RGS2 protein. The N-terminal region of

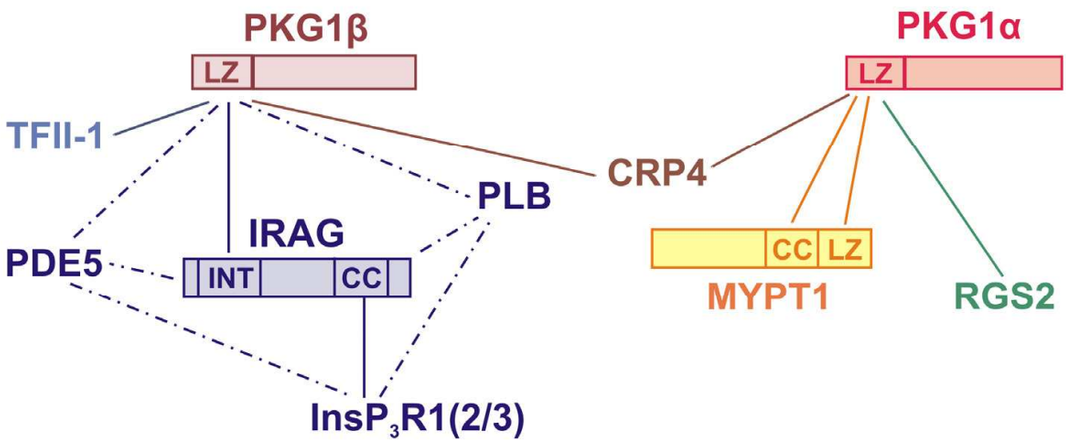



Fig. 2. PKG1α and PKG1β complexes identified by affinity chromatography methods
The filled lines indicate confirmed stable interactions between two complex proteins. When the respective interaction sites are known they are illustrated in the figure. The broken lines indicate putative interactions of complex proteins.
*Complex proteins:* PKG1α/β: cGMP-dependent protein kinase 1α/β; IRAG: inositol-trisphosphate receptor-associated cGMP-kinase substrate protein; $InsP_3R1/2/3$: inositol 1,4,5-trisphophate 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 PKG1β; CC: coiled-coil domain

RGS2 (GST-RGS2-N; residues 1-79) bound endogenous PKG1α 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 PKG1α in vascular smooth muscle cells (Fig. 2).

GST-fusion proteins were also generated to investigate the MYPT1-PKG1α 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 PKG1α. After that, it was demonstrated with GST-fusion proteins of the N-termini of PKG1α (GST PKG1α (1-59)), PKG1β (GST PKG1β (1-92)) and PKG2 (GST PKG2 (1-272)) that only PKG1α interacts with MYPT1 (Fig. 2). Subsequently, the relevance of the leucine zipper of MYPT1 for the interaction with PKG1α 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 1007A1014A mutant and MYPT1 1021A1028A mutant). Only the wild-type GST-MYPT1 bound endogenous PKG1α 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 $CT_{181}$ L1021A/L1028A and GST MYPT1 $CT_{181}$ L1007A/L1014A) did not bind MYPT1 from VSMC lysate (Surks & Mendelsohn, 2003). On the other hand the PKG1α leucine/isoleucine zipper

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

Table 2. Interaction of GST- and His-tagged fusion proteins (PKG1α, PKG1β 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 PKG1α and endogenous PKG1α 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 PKG1α-MYPT1 complex. Therefore, the interaction between the leucine zipper of PKG1α ($PKG1\alpha_{1-59}$) and the C-terminal CC domain of the Myosin Binding Subunit was tested (Sharma et al., 2008). A C-terminal $His_6$-MYPT1 $CT_{100}$ (residues 930-1030) protein which was bound to $Ni^{2+}$-NTA resin precipitated GST-$PKG1\alpha_{1-59}$. In isothermal titration calorimetry studies and NMR experiments the importance of the residues 929-970 (MYPT1 $CT_{42}$) for the interaction with the leucine zipper of PKG1α was demonstrated (Sharma et al., 2008). In short, if the leucine-zipper motif of MYPT1 is absent, the PKG1α 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 PKG1β. A GST-tagged full-length PKG1β 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 PKG1α 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 PKG1β was tested. Acidic residues in the N-terminal sequence of PKG1β 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 PKG1β (GST PKG1β (1-110) E29K and GST PKGIβ (1-110) D26K/E31R) showed no longer binding to IRAG and therefore it was concluded that acidic residues in PKG1β 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 PKG1β. 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 PKG1β 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 PKG1β 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 PKG1β homodimerization.

The importance of PKG1 homodimerization was demonstrated by the cardiovascular deficits exhibited by transgenic mice expressing a dimerization-deficient form of PKG1α (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 PKG1β dimerization/docking domain by investigating a His-tagged PKG1β D/D domain (Casteel et al., 2011) (described in 2.).

Similarly to the IRAG-PKG1β interaction, the interaction between PKG1β 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 PKG1β but not by PKG1α. It was shown that electrostatic interactions are important for binding of PKG1β to GST-TFII-I, because high NaCl concentrations (400 mM) disrupted binding. Acidic residues in the PKG1β leucine zipper are

important for the interaction with basic amino acids of TFII-I which was proved by analysis of GST-tagged PKG1β 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 PKG1α and PKG1β 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 phosporylation 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 PKG1β/IRAG/$InsP_3R1$ 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 PKG1β and $InsP_3R1$ is destroyed by IRAG deletion and that the $InsP_3R1$ misses a stable interaction site for PKG1β.

In 2003, Phospholamban (PLB) was identified as a component of the PKG1β/IRAG/$InsP_3R1$ 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 $InsP_3R1$, IRAG and PKG1β, 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-$InsP_3R1$, anti-IRAG, anti-PKG1 and anti-PLB antibody. In each case, the autoradiogram showed phosphorylated $InsP_3R1$, IRAG and PKG1β which is a further indication that PLB is a component of the macrocomplex and interacts with PKG1β.

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. PKG1β, PKG2, PDE2A and PDE5A were identified as primary and IRAG, $InsP_3R1$ and $InsP_3R2$ as secondary interactors. After CRP stimulation, the secondary interactors showed increased enrichment versus control whereas binding of PKG1β 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 PKG1β 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-PKGIβ and the ternary PKG macrocomplex

IRAG is a 125 kDa substrate of PKG1β 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 PKG1β but not with PKG1α. Two arginines of the IRAG interaction domain (R124 and R125) are essential for the electrostatic interaction with acidic aa of PKG1β (D26, E29, E31) (Casteel et al., 2005). PKG1β and IRAG form a ternary complex together with $InsP_3R1$ in smooth muscle, platelets and osteoclasts. IRAG is the core component of the complex which interacts with the $InsP_3R1$ by its central coiled-coil domain. Deletion of this coiled-coil domain prevent the $InsP_3R1$ interaction but does not alter IRAG-PKG1β binding. However, the deficiency of IRAG in IRAG-KO mice destructs the ternary complex showing that $InsP_3R1$ does not stably interact with PKG1β. 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 $InsP_3R$ isoforms, namely $InsP_3R2$ and $InsP_3R3$ (Masuda et al., 2010). The exact mechanism of IRAG interaction with $InsP_3R$ and the inhibition of $InsP_3R$ gating was not established so far and remains a fundamental subject of IRAG function.

### 4.2 MYPT1-PKG1α

PKG1α interaction with MYPT1, a 130 kDa myosin-binding subunit of phosphatase 1, was revealed by co-immunopreciptation, two hybrid analysis and mutagenesis studies. The Ile/Leu-zipper domain of PKG1α (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 PKG1α 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 PKG1α through its N1-N2 coiled-coil domain but is cGMP-insensitive (Payne et al., 2006).

### 4.3 RGS2-PKG1α

The PKG1α-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 PKG1α at the plasma membrane was enhanced by RGS2 phosphorylation suggesting a thereby stabilized complex of these proteins. Thus, RGS2 is activated by PKG1α and hence $G_q$-triggered $InsP_3$-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 PKG1α-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 PKG1α- and PKG1β-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 PKG1β 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 PKG1β 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 PKG1β (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 PKG1α and PKG1β 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 immunogical 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.

## 6. Acknowledgement

Work done in the authors laboratory was supported by DFG, GRK760 and SFB699. Tobias Holzammer aided in the art work.

## 7. References

Aitken, A., Hemmings, B.A. & Hofmann, F. (1984) Identification of the residues on cyclic GMP-dependent protein kinase that are autophosphorylated in the presence of cyclic AMP and cyclic GMP. *Biochim Biophys Acta*, 790, 219-225.

Ammendola, A., Geiselhoringer, A., Hofmann, F. & Schlossmann, J. (2001) Molecular determinants of the interaction between the inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and cGMP kinase Ibeta. *J Biol Chem*, 276, 24153-24159.

Antl, M., von Bruhl, M.L., Eiglsperger, C., Werner, M., Konrad, I., Kocher, T., Wilm, M., Hofmann, F., Massberg, S. & Schlossmann, J. (2007) IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. *Blood*, 109, 552-559.

Casteel, D.E., Boss, G.R. & Pilz, R.B. (2005) Identification of the interface between cGMP-dependent protein kinase Ibeta and its interaction partners TFII-I and IRAG reveals a common interaction motif. *J Biol Chem*, 280, 38211-38218.

Casteel, D.E., Smith-Nguyen, E.V., Sankaran, B., Roh, S.H., Pilz, R.B. & Kim, C. (2011) A crystal structure of the cyclic GMP-dependent protein kinase I{beta} dimerization/docking domain reveals molecular details of isoform-specific anchoring. *J Biol Chem*, 285, 32684-32688.

Casteel, D.E., Zhuang, S., Gudi, T., Tang, J., Vuica, M., Desiderio, S. & Pilz, R.B. (2002) cGMP-dependent protein kinase I beta physically and functionally interacts with the transcriptional regulator TFII-I. *J Biol Chem*, 277, 32003-32014.

Castro, L.R., Schittl, J. & Fischmeister, R. (2010) Feedback control through cGMP-dependent protein kinase contributes to differential regulation and compartmentation of cGMP in rat cardiac myocytes. *Circ Res*, 107, 1232-1240.

Desch, M., Sigl, K., Hieke, B., Salb, K., Kees, F., Bernhard, D., Jochim, A., Spiessberger, B., Hocherl, K., Feil, R., Feil, S., Lukowski, R., Wegener, J.W., Hofmann, F. &

Schlossmann, J. (2010) IRAG determines nitric oxide- and atrial natriuretic peptide-mediated smooth muscle relaxation. *Cardiovasc Res*, 86, 496-505.

Dostmann, W.R., Taylor, M.S., Nickl, C.K., Brayden, J.E., Frank, R. & Tegge, W.J. (2000) Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase Ialpha inhibit NO-induced cerebral dilation. *Proc Natl Acad Sci U S A*, 97, 14772-14777.

Geiselhoringer, A., Werner, M., Sigl, K., Smital, P., Worner, R., Acheo, L., Stieber, J., Weinmeister, P., Feil, R., Feil, S., Wegener, J., Hofmann, F. & Schlossmann, J. (2004) IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase. *Embo J*, 23, 4222-4231.

Given, A.M., Ogut, O. & Brozovich, F.V. (2007) MYPT1 mutants demonstrate the importance of aa 888-928 for the interaction with PKGIalpha. *Am J Physiol Cell Physiol*, 292, C432-439.

Haug, L.S., Jensen, V., Hvalby, O., Walaas, S.I. & Ostvold, A.C. (1999) Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic nucleotide-dependent kinases in vitro and in rat cerebellar slices in situ. *J Biol Chem*, 274, 7467-7473.

Hofmann, F., Bernhard, D., Lukowski, R. & Weinmeister, P. (2009) cGMP regulated protein kinases (cGK). *Handb Exp Pharmacol*, 137-162.

Hofmann, F., Feil, R., Kleppisch, T. & Schlossmann, J. (2006) Function of cGMP-dependent protein kinases as revealed by gene deletion. *Physiol Rev*, 86, 1-23.

Huang, Q.Q., Fisher, S.A. & Brozovich, F.V. (2004) Unzipping the role of myosin light chain phosphatase in smooth muscle cell relaxation. *J Biol Chem*, 279, 597-603.

Huber, A., Neuhuber, W.L., Klugbauer, N., Ruth, P. & Allescher, H.D. (2000) Cysteine-rich protein 2, a novel substrate for cGMP kinase I in enteric neurons and intestinal smooth muscle. *J Biol Chem*, 275, 5504-5511.

Johnson, A.D., Yanek, L.R., Chen, M.H., Faraday, N., Larson, M.G., Tofler, G., Lin, S.J., Kraja, A.T., Province, M.A., Yang, Q., Becker, D.M., O'Donnell, C.J. & Becker, L.C. (2010) Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. *Nat Genet*, 42, 608-613.

Kaboord, B. & Perr, M. (2008) Isolation of proteins and protein complexes by immunoprecipitation. *Methods Mol Biol*, 424, 349-364.

Kemp, B.E. & Pearson, R.B. (1990) Protein kinase recognition sequence motifs. *Trends Biochem Sci*, 15, 342-346.

Kim, J.J., Casteel, D.E., Huang, G., Kwon, T.H., Ren, R.K., Zwart, P., Headd, J.J., Brown, N.G., Chow, D.C., Palzkill, T. & Kim, C. (2011) Co-Crystal Structures of PKG Ibeta (92-227) with cGMP and cAMP Reveal the Molecular Details of Cyclic-Nucleotide Binding. *PLoS One*, 6, e18413.

Klaiber, M., Kruse, M., Volker, K., Schroter, J., Feil, R., Freichel, M., Gerling, A., Feil, S., Dietrich, A., Londono, J.E., Baba, H.A., Abramowitz, J., Birnbaumer, L., Penninger, J.M., Pongs, O. & Kuhn, M. (2010) Novel insights into the mechanisms mediating the local antihypertrophic effects of cardiac atrial natriuretic peptide: role of cGMP-dependent protein kinase and RGS2. *Basic Res Cardiol*, 105, 583-595.

Koller, A., Schlossmann, J., Ashman, K., Uttenweiler-Joseph, S., Ruth, P. & Hofmann, F. (2003) Association of phospholamban with a cGMP kinase signaling complex. *Biochem Biophys Res Commun*, 300, 155-160.

Lalli, M.J., Shimizu, S., Sutliff, R.L., Kranias, E.G. & Paul, R.J. (1999) [Ca2+]i homeostasis and cyclic nucleotide relaxation in aorta of phospholamban-deficient mice. *Am J Physiol*, 277, H963-970.

Lee, E., Hayes, D.B., Langsetmo, K., Sundberg, E.J. & Tao, T.C. (2007) Interactions between the leucine-zipper motif of cGMP-dependent protein kinase and the C-terminal region of the targeting subunit of myosin light chain phosphatase. *J Mol Biol*, 373, 1198-1212.

Margarucci, L., Roest, M., Preisinger, C., Bleijerveld, O.B., van Holten, T.C., Heck, A.J. & Scholten, A. (2011) Collagen stimulation of platelets induces a rapid spatial response of cAMP and cGMP signaling scaffolds. *Mol Biosyst*, DOI: 10.1039.

Masuda, W., Betzenhauser, M.J. & Yule, D.I. (2010) InsP3R-associated cGMP kinase substrate determines inositol 1,4,5-trisphosphate receptor susceptibility to phosphoregulation by cyclic nucleotide-dependent kinases. *J Biol Chem*, 285, 37927-37938.

Michael, S.K., Surks, H.K., Wang, Y., Zhu, Y., Blanton, R., Jamnongjit, M., Aronovitz, M., Baur, W., Ohtani, K., Wilkerson, M.K., Bonev, A.D., Nelson, M.T., Karas, R.H. & Mendelsohn, M.E. (2008) High blood pressure arising from a defect in vascular function. *Proc Natl Acad Sci U S A*, 105, 6702-6707.

Obst, M., Tank, J., Plehm, R., Blumer, K.J., Diedrich, A., Jordan, J., Luft, F.C. & Gross, V. (2006) NO-dependent blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol*, 290, R1012-1019.

Osborne, B.W., Wu, J., McFarland, C.J., Nickl, C.K., Sankaran, B., Casteel, D.E., Woods, V.L., Jr., Kornev, A.P., Taylor, S.S. & Dostmann, W.R. (2011) Crystal Structure of cGMP-Dependent Protein Kinase Reveals Novel Site of Interchain Communication. *Structure*, 19, 1317-1327.

Payne, M.C., Zhang, H.Y., Prosdocimo, T., Joyce, K.M., Koga, Y., Ikebe, M. & Fisher, S.A. (2006) Myosin phosphatase isoform switching in vascular smooth muscle development. *J Mol Cell Cardiol*, 40, 274-282.

Roy, A.L. (2001) Biochemistry and biology of the inducible multifunctional transcription factor TFII-I. *Gene*, 274, 1-13.

Ruth, P., Pfeifer, A., Kamm, S., Klatt, P., Dostmann, W.R. & Hofmann, F. (1997) Identification of the amino acid sequences responsible for high affinity activation of cGMP kinase Ialpha. *J Biol Chem*, 272, 10522-10528.

Rybalkin, S.D., Rybalkina, I.G., Feil, R., Hofmann, F. & Beavo, J.A. (2002) Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J Biol Chem*, 277, 3310-3317.

Schinner, E., Salb, K. & Schlossmann, J. (2011) Signaling via IRAG is essential for NO/cGMP-dependent inhibition of platelet activation. *Platelets*.

Schlossmann, J., Ammendola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., Wang, G.X., Allescher, H.D., Korth, M., Wilm, M., Hofmann, F. & Ruth, P. (2000)

Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature*, 404, 197-201.

Schlossmann, J. & Desch, M. (2009) cGK substrates. *Handb Exp Pharmacol*, 163-193.

Schlossmann, J. & Desch, M. (2011) IRAG and Novel PKG Targeting in the cardiovascular system. *Am J Physiol Heart Circ Physiol*, 301, H672-H682.

Schmidtko, A., Gao, W., Sausbier, M., Rauhmeier, I., Sausbier, U., Niederberger, E., Scholich, K., Huber, A., Neuhuber, W., Allescher, H.D., Hofmann, F., Tegeder, I., Ruth, P. & Geisslinger, G. (2008) Cysteine-rich protein 2, a novel downstream effector of cGMP/cGMP-dependent protein kinase I-mediated persistent inflammatory pain. *J Neurosci*, 28, 1320-1330.

Sharma, A.K., Zhou, G.P., Kupferman, J., Surks, H.K., Christensen, E.N., Chou, J.J., Mendelsohn, M.E. & Rigby, A.C. (2008) Probing the interaction between the coiled coil leucine zipper of cGMP-dependent protein kinase Ialpha and the C terminus of the myosin binding subunit of the myosin light chain phosphatase. *J Biol Chem*, 283, 32860-32869.

Sun, X., Kaltenbronn, K.M., Steinberg, T.H. & Blumer, K.J. (2005) RGS2 is a mediator of nitric oxide action on blood pressure and vasoconstrictor signaling. *Mol Pharmacol*, 67, 631-639.

Surks, H.K. & Mendelsohn, M.E. (2003) Dimerization of cGMP-dependent protein kinase 1alpha and the myosin-binding subunit of myosin phosphatase: role of leucine zipper domains. *Cell Signal*, 15, 937-944.

Surks, H.K., Mochizuki, N., Kasai, Y., Georgescu, S.P., Tang, K.M., Ito, M., Lincoln, T.M. & Mendelsohn, M.E. (1999) Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Ialpha. *Science*, 286, 1583-1587.

Tang, K.M., Wang, G.R., Lu, P., Karas, R.H., Aronovitz, M., Heximer, S.P., Kaltenbronn, K.M., Blumer, K.J., Siderovski, D.P., Zhu, Y. & Mendelsohn, M.E. (2003) Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. *Nat Med*, 9, 1506-1512.

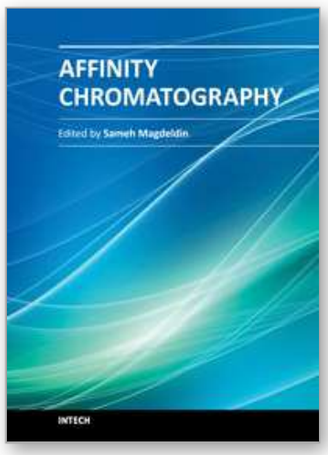
Wen, Y.D., Cress, W.D., Roy, A.L. & Seto, E. (2003) Histone deacetylase 3 binds to and regulates the multifunctional transcription factor TFII-I. *J Biol Chem*, 278, 1841-1847.

Wilson, L.S., Elbatarny, H.S., Crawley, S.W., Bennett, B.M. & Maurice, D.H. (2008) Compartmentation and compartment-specific regulation of PDE5 by protein kinase G allows selective cGMP-mediated regulation of platelet functions. *Proc Natl Acad Sci U S A*, 105, 13650-13655.

Wooldridge, A.A., MacDonald, J.A., Erdodi, F., Ma, C., Borman, M.A., Hartshorne, D.J. & Haystead, T.A. (2004) Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides. *J Biol Chem*, 279, 34496-34504.

Yaroslavskiy, B.B., Turkova, I., Wang, Y., Robinson, L.J. & Blair, H.C. (2010) Functional osteoclast attachment requires inositol-1,4,5-trisphosphate receptor-associated cGMP-dependent kinase substrate. *Lab Invest*, 90, 1533-1542.

Zhang, T., Zhuang, S., Casteel, D.E., Looney, D.J., Boss, G.R. & Pilz, R.B. (2007) A cysteine-rich LIM-only protein mediates regulation of smooth muscle-specific gene expression by cGMP-dependent protein kinase. *J Biol Chem*, 282, 33367-33380.




**Affinity Chromatography**

Edited by Dr. Sameh Magdeldin

ISBN 978-953-51-0325-7
Hard cover, 368 pages
**Publisher** InTech
**Published online** 21, March, 2012
**Published in print edition** March, 2012

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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