Molecular Determinants of the Interaction between the Inositol 1,4,5-Trisphosphate Receptor-associated cGMP Kinase Substrate (IRAG) and cGMP Kinase Iβ

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From the Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedaster Straße 29, D-80802 München, Germany

Aldo Ammendola‡, Angela Geiselhöringer‡, Franz Hofmann, and Jens Schlossmann§

Cyclic GMP-dependent protein kinase I (cGKI) affects the inositol 1,4,5-trisphosphate (InsP₃)−dependent release of intracellular calcium by phosphorylation of IRAG (inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate). IRAG is present in a macromolecular complex with the InsP₃ receptor type I (InsP₃RI) and cGKI. The specificity of the interaction between these three proteins was investigated by using the yeast two-hybrid system and by co-precipitation of expressed proteins. The amino-terminal region containing the leucine zipper (amino acids 1−53) of cGKI but not that of cGKI or cGKII interacted with the sequence between amino acids 152 and 184 of IRAG in vitro and in vivo most likely through electrostatic interaction. cGKIβ did not interact with the InsP₃RI, but co-precipitated the InsP₃RI in the presence of IRAG indicating that IRAG bound to the InsP₃RI and to cGKI. cGKIβ phosphorylated up to four serines in IRAG. Mutation of these four serines to alanine showed that cGKIβ−dependent phosphorylation of Ser⁶⁹⁶ is necessary to decrease calcium release from InsP₃-sensitive stores. These results show that cGMP induced reduction of cytosolic calcium concentrations requires cGKIβ and phosphorylation of Ser⁶⁹⁶ of IRAG.

Signal transduction via NO/cGMP/cGKI is involved in a variety of cellular mechanisms including smooth muscle contractility and platelet aggregation (1−4). cGKI affects smooth muscle tone by either decreasing the release of calcium from InsP₃-sensitive stores (5−9) or by reducing calcium sensitivity of the contractile elements (10, 11). During the last years, several mechanisms were proposed for the action of cGKI mediating these effects. A decrease of the cytosolic calcium concentration by cGKI might involve reduced InsP₃ synthesis (12−15), enhanced calcium re-uptake by intracellular stores via CaATPase (16), or inhibition of calcium release via the InsP₃R (17). The molecular mechanisms for these different possible intracellular calcium regulation pathways were only partly resolved up to now.

Recently, we identified a 125-kDa cGKI substrate protein which was designated as inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG). IRAG which is phosphorylated by cGKI is associated in a macromolecular complex with cGKIβ and InsP₃RI in smooth muscle (9). The observed perinuclear localization of heterologously expressed IRAG suggested the potential role of IRAG as a modulator of calcium release from intracellular stores. Indeed, functional studies revealed that IRAG inhibits InsP₃-induced calcium release after activation of cGKIβ with 8-pCPT-cGMP in COS-7 cells (9). However, the precise mechanism by which IRAG influences calcium release is still unknown.

In the present study we investigated the molecular determinants for the interaction of IRAG and cGKI. It is shown that IRAG interacts specifically with the amino-terminal region containing the leucine zipper of cGKIβ. Phosphorylation of Ser⁶⁹⁶ of IRAG is essential for the inhibition of InsP₃−induced calcium release.

EXPERIMENTAL PROCEDURES

Materials—The yeast strain EGY48 (MATa, his3, trp1, ura3, lex-Aout, Leu2) and the yeast expression plasmids pEG202, pJG4-5, and pSH18-34 were used for the two-hybrid screen. Yeast media and dropout media lacking the appropriate amino acids were obtained from CLONTECH (Heidelberg, Germany) and Difco (Hamburg, Germany), respectively. The full-length rat cDNAs of the neuronal InsP₃RI (S1/S2+) (18) and the peripheral InsP₃RI (S1−/S2−) (19) were a gift from Dr. Ilya Bezprozvanny (Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX).

Antibodies—A polyclonal antibody specific for IRAG, raised in rabbits against recombinant IRAGc⁰−⁹⁰ expressed in bacteria, was used for Western blot analysis at a dilution of 1:1000. Further antibodies were directed against cGKI (8) and InsP₃RI (ABR Biochemicals).

Plasmid Construction of Baits and Preys—The bovine IRAG cDNA was the template of all IRAG constructs (baits) which were synthesized by PCR. The generated IRAG amplicons were purified and inserted into the BamHI/EcoRI-digested expression plasmid pEG202 (20) in-frame with the DNA-binding domain of LexA, yielding pEG202-LexA/IRAG. The full-length and truncated PCR amplicons of bovine cGKια and cGKIβ were ligated into the EcoRI/Xhol sites of the pJG4-5 expression plasmid (20) and used as preys. The inserts were fused in-frame to the "acid loop" DNA activation domain of pJG4-5, yielding pJG4-5/LexA/IRAG. As cGKI Prgk2 gene from mouse contains an internal Xhol site it was subcloned as EcoRI fragment into pJG4-5. The correct orientation was checked by sequence analysis. The full-length rat InsP₃RI (S1+−/S2+−) without the channel forming domain was divided into five different fragments. These inserts were amplified by PCR and ligated into pJG4-5 via EcoRI and Xhol in order to obtain various pJG4-5/InsP₃RI constructs. cGKIβ was generated as bait through EcoRI/Xhol digestion.
Phosphorylation of IRAG by cGKIβ

Measurement of Intracellular Calcium—COS-7 cells were loaded with Fura-2 AM (5 μM, ICN) before measuring [Ca2+]i, by the dual-wavelength microfluorescence technique (9). Analysis of the area under the curve of calcium transients was performed using the software program Microcal® Origin 6.0.

RESULTS

IRAG Interacts with the Amino-terminal Region Containing the Leucine Zipper of cGKIβ—IRAG is a new compound of the NO/cGMP signaling pathway which, in association with InsP3R and cGKIβ, negatively regulates InsP3-induced calcium release. It has been shown that IRAG and InsP3R are phosphorylated after addition of 8-cPCT-cGMP by cGKI. IRAG, InsP3R, and cGKI associate to a multimeric complex which has been purified from microsomal membranes of bovine tracheal smooth muscle (9). To analyze the interaction and identify the interaction sites of IRAG with cGK, a yeast two-hybrid screen was performed using an IRAG derivative (IRAG53–845) lacking the putative NH2- and COOH-terminal transmembrane domains as bait and cGKI variants and cGKII as preys (Fig. 1). Strong interaction was observed between IRAG and cGKIβ but no detectable interaction between IRAG and cGKIα or cGKIβ (Fig. 1, A and B). Further analysis showed that cGKβ interacted with its amino terminus with IRAG. This is of interest since the Iα and Iβ isozymes of cGKI differ in their first 100 amino acids (23). Within the NH2-terminal region of cGKIβ, only the part containing the leucine zipper (cGKβ53–85: cGKβ-NT (1)) but not the linker (cGKβ53–104: cGKβ-NT (2)) showed association with IRAG (Fig. 1B). Next, we analyzed which part of IRAG interacts with cGKβ. For this study different IRAG variants were used as baits and full-length cGKIβ or cGKβ variants as prey in the yeast two-hybrid system (Fig. 1, A and C). The amino-terminal region containing the leucine zipper of cGKIβ interacted with the peptide sequence between amino acids 152 and 184 of IRAG. This sequence of IRAG contains 33 amino acids of which 16 amino acids are charged (Asp, Glu, Lys, and Arg). The corresponding peptide of cGKβ has 22 charged amino acids located between the leucine/isoleucine residues of the cGKβ leucine zipper (Fig. 1D). Therefore, interaction between these two sites might be mediated by electrostatic interactions. Interestingly, the putative coiled-coil domain present in the IRAG protein is not involved in the complex formation between IRAG and cGKIβ suggesting that this site might mediate the assembly of IRAG with the InsP3R.

IRAG Mediates the Assembly of cGKIβ and InsP3R in a Macrocomplex—The interpretations of the two-hybrid system experiments were supported by the results obtained after in vivo expression of the various proteins in COS-7 cells. The two different cGKI isoforms, cGKIα and cGKIβ, were separately coexpressed with full-length IRAG and/or InsP3R. Using partially purified or pure proteins as standard (for example, see Fig. 2a in Ref. 9), we estimated that cGKIβ, IRAG, and/or InsP3R were expressed at approximately equal amounts. Phosphorylation of IRAG and co-precipitation of the proteins was studied in cell lysates (Fig. 1E). IRAG was phosphorylated heavily in the presence of cGKIβ and very weak or not at all in the presence of cGKIα. Furthermore, cGMP-agarose co-precipitated only IRAG and cGKIβ but not IRAG and cGKIα. The inability of cGKIα to interact with IRAG was caused by its inability to bind the IRAG protein, since regular Western blots showed that each protein was expressed to a similar level in the COS-7 cells. From these results we concluded that phosphorylation of IRAG requires interaction of the amino-terminal region containing the leucine zipper of cGKIβ with IRAG.

Next we investigated the association of cGKβ with the InsP3R, since the InsP3R is phosphorylated by cGKI (24, 25) and is present in the cGKI-IRAG macrocomplex. The InsP3R was coexpressed with cGKβ in the absence of IRAG in COS-7
cells. cGMP-agarose did not co-precipitate InsP_3RI with cGKI β (Fig. 1E). This result agreed with a two-hybrid screen using cGKI β as bait and different InsP_3RI fragments as preys. In this screen no interaction between these proteins could be detected (data not shown). Therefore, these results indicate that cGKI and InsP_3RI are not stably associated with each other. However, when IRAG was expressed together with cGKI β and InsP_3RI all three proteins were co-precipitated (Fig. 1E). 90%
or more of the solubilized coexpressed proteins were bound to the cGMP-agarose in the presence of cGKIβ. These results suggest that IRAG could mediate the assembly of these proteins in a macrocomplex.

cGKIβ Phosphorylates IRAG Predominantly at Ser696—Several potential cGKI phosphorylation sites have been identified previously within the IRAG protein (Fig. 1C) (9). In the present study, the functional role of the phosphorylation of these serine residues by cGKI was analyzed. In control experiments, both native IRAG and expressed IRAGα, which is 10 kDa larger than the native IRAG (see Ref. 9) were phosphorylated by cGKIβ in the presence of 8-pCPT-cGMP in the cell lysates (Fig. 2A). Similar to the expression level, the stoichiometry for cGKIβ and IRAG was approximately equal in COS-7 cells. This phosphorylation reaction was specific for cGK, since cGMP-dependent phosphorylation of IRAGα was only observed when cGKIβ was coexpressed with IRAG (Fig. 2A). The COS-7 expression system was used next to study the phosphorylation efficiency of the single phosphorylation sites. For this purpose, the various serine phosphorylation sites (at positions 118, 629, 683, and 696) were mutated to alanine (S118A, S629A, S683A, S4A) by PCR. The single mutants and several multiple mutants were transiently expressed in COS-7 cells together with cGKIβ and phosphorylation of these mutant proteins by cGKIβ was analyzed compared with wild type IRAG (Fig. 2B). Mutation of Ser696 to alanine (S4A) diminished significantly cGMP-dependent phosphorylation of IRAG to 53.1 ± 5.9% (n = 5) when compared with the phosphorylation of the wild type protein (Fig. 2B). In contrast, the phosphorylation efficiency of the triple mutant S123A was not affected being 126.1 ± 12.9% (n = 5). As expected, a time course of the phosphorylation indicated that the phosphorylation reaction was maximal between 1 and 2 min (Fig. 2C). The IRAG S4A mutant protein incorporated about half the amount of...
phosphate compared with the wild type or the IRAG S123A mutant protein supporting the notion that Ser696 is a major cGKIβ phosphorylation site in IRAG.

The reduced phosphorylation of IRAG S4A was neither caused by a decreased expression of the mutant IRAG protein nor by a reduced affinity of cGKIβ for the mutant proteins (Fig. 3). The extent of co-precipitation of cGKIβ and IRAG was analyzed using cGMP affinity chromatography followed by phosphorylation and immunoblot analysis. No difference could be observed in the amount of IRAG wild type or mutant proteins co-precipitated with cGKIβ, indicating that the mutation of the various serine residues did not affect the interaction of IRAG with cGKIβ. This result was expected since the interaction site of IRAG did not include any of the mutated serines. However, phosphorylation was significantly reduced in the S4A mutant protein (Fig. 3). These results suggest that Ser696 is the predominant cGKIβ phosphorylation site.

**Phosphorylation of Ser696 Mediates cGMP-dependent Inhibition of Calcium Release**—Phosphorylation of IRAG by cGKI inhibits bradykinin- and InsP3-induced calcium release from intracellular stores in COS-7 cells (9). The phosphorylation site(s) responsible for the inhibition were not identified. We anticipated that phosphorylation of the above mutated serines were involved in this functional effect of cGKIβ. Therefore, we tested whether or not cGMP-dependent inhibition of the calcium release was observed after transfection of the mutated IRAG constructs into COS-7 cells. Western blot analysis indicated that wild type IRAG and all mutated proteins were expressed to the same level in COS-7 cells.

Fig. 4, A and B, show representative traces and the statistics, respectively, of bradykinin-induced calcium transients of COS-7 cells transfected with various IRAG mutants (S4A, S123A, and S1234A) and cGKIβ. cGKIβ was activated by addition of 8-pCPT-cGMP before the second stimulation with bradykinin. In cells expressing IRAG mutants containing the S4A mutation (S4A and S1234A), cGKIβ was unable to inhibit the calcium release. In contrast, active cGKIβ decreased to a similar extent the second Ca2+ peak in cells expressing IRAG containing the mutated serines S123A or wild type IRAG (Fig. 4).

The calculated area under the curve ratio of second to first bradykinin-induced calcium transient in COS-7 cells transfected with cGKIβ and pEGFP-IRAG wild type or mutated variants. Transfected cells were loaded with FURA-2 AM. For details of the loading procedure and recording of the FURA-2 signal see “Experimental Procedures.” Cells were first stimulated with bradykinin (100 nM in the pipette) for 10 s. The cells were then superfused with buffer for 10 min (gap), the last 5 min with 100 μM 8-pCPT-cGMP (cGMP), followed by a second stimulation with bradykinin. A, representative calcium transients of IRAG mutants S4A, S123A, and S1234A are shown. The arrow marks the start of the bradykinin superfusion. The horizontal bars indicate 20 s and 200 nM Δ[Ca2+]i, respectively. B, statistical analysis of single (upper panel) and multiple (lower panel) IRAG mutants. The ratio of the area under the curve (area under the curve ratio) of second to first transient is shown. Values are x ± S.E. (number of cells are indicated within the columns). *, significantly different against wild type at p < 0.001; §, significantly different against S4A at p < 0.001.

**DISCUSSION**

cGK has been implicated in various physiological signal transduction pathways leading to relaxation of smooth muscle,
inhibition of platelet aggregation, cell motility and cell proliferation, secretion of intestinal fluid, bone growth, renin secretion, guidance of nerve fibers, and the development of synaptic plasticity (1–4, 26). Part of these functions are regulated by different cGK enzymes as shown by deletion of the gene for cGKII and cGKI (8, 27). Only few proteins have been identified that are phosphorylated by cGKI or -II and are involved in the regulation of the proposed functions. These proteins include cGMP hydrolyzing phosphodiesterase 5 (28), the large subunit of the maxi-K channel (29, 30), CRP2 (31), telokin (32), VASP (33), CFTR (27, 34), the cerebellar G-substrate (35, 36) and IRAG (9). There are additional proteins such as the myosin binding subunit of phosphatase I (37, 38) and Rhα (39) that may be in vivo substrates for cGKI and affect smooth muscle tone in the absence of elevated cytosolic Ca$^2^+$ concentrations.

IRAG has been identified recently as the substrate for cGKI that mediates cGMP-dependent relaxation of smooth muscle by decreasing the calcium release from InsP$_3$-sensitive stores (9). IRAG was co-purified together with InsP$_3$R and cGKI. In this study, we show that IRAG interacts only with the amino-terminal region containing the leucine zipper of cGKI and not with that of cGKIs or cGKII. The IRAG sequence interacting with the amino-terminal region containing the leucine zipper of cGKI does not contain a phosphorylation site supporting the notion that interaction between cGKB and IRAG is independent of the mechanism allowing substrate binding. This interaction does not require activation of the kinase domain and should be stable in relaxed and contracted cells. Localization of cGKI to IRAG explains the observed phosphorylation specificity for the β isozyme and the inability of cGKIα to modify IRAG.

The results of this study contribute to earlier observations that cGMP kinases recognize their in vivo substrates by interaction of amino-terminal kinase sequences with the substrate protein. This mechanism has been identified not only for the co-localization of cGKIβ and IRAG, but was found also with cGKII and cGKIα. Myristoylation of the first glycine of cGKII is required for membrane localization of the enzyme and the phosphorylation of CFTR (34, 40). Furthermore, it has been shown that cGKIα binds with its unique amino-terminal leucine zipper to the myosin binding subunit of phosphatase I (38), the skeletal muscle troponin T (41), and the male germ cell-specific 42-kDa protein GKP42 (42). In each case it appears that the kinase binds to a substrate sequence that is outside the phosphorylation site. The permanent localization of the kinase to its substrate allows a rapid response after increases in cGMP. In comparison to the cAMP kinase system it is obvious that the subcellular organization of the cGMP kinases does not require extra anchoring proteins as necessary for the cAMP kinase signaling system (43).

IRAG, cGKBβ, and the InsP$_3$-RI could be co-purified together, whereas cGKBβ and InsP$_3$RI were not co-precipitated and did not interact in the two-hybrid screen. It is therefore plausible to assume that the assembly of the triple complex required expression of IRAG. In support of this hypothesis is the finding that the InsP$_3$-RI phosphorylation was evident only when cGKIβ was coexpressed with the InsP$_3$RI. The differential possibility of cGKI to phosphorylate the InsP$_3$RI is in good agreement with in vivo findings. cGKI phosphorylated in vivo the smooth muscle InsP$_3$RI (24) and affected calcium release in megakaryocytes (17). This is not in contrast to the above results, since smooth muscle and platelets express high levels of cGKIβ and IRAG which apparently allows cGKIβ-dependent phosphorylation of IRAG and the InsP$_3$RI. Possibly, additional substrates exist for cGKIβ in smooth muscle since ~50% of cGKIβ immunoreactivity has been found in the cytosolic fraction (44).

Phosphorylation of the InsP$_3$RI by cGKIβ apparently did not contribute to the decrease in calcium release. This effect depended on the phosphorylation of Ser$^{696}$ of IRAG. Mutation of Ser$^{696}$ to alanine abolished complete the modulatory effect of cGKIβ on calcium release without disruption of the triple complex. The phosphorylation of the InsP$_3$-RI by CAMP kinase may be important to modulate the calcium release in vivo (45) and may be an independent possibility to regulate smooth muscle contractility. Additional experiments are necessary to clarify whether or not cAMP kinase modulates smooth muscle contraction via phosphorylation of the InsP$_3$-RI and requires the presence of IRAG. It is quite possible that modulation of the InsP$_3$-dependent calcium release is specifically caused by cGKIβ, since CAMP analogs were unable to modulate the calcium release in murine aortic wild type and cGKI negative smooth muscle cells (8).

Together with the functional data obtained by intracellular calcium measurements our results clearly demonstrate that phosphorylation of Ser$^{696}$ is indispensable for the inhibitory effect of IRAG and cGKI on InsP$_3$-induced calcium release. The data presented here add a further functional element how the NO/cGMP/cGKI signaling pathway is involved in calcium regulation. With the identification of the functional phosphorylation site of the cGKIβ substrate protein IRAG we threw more light on the molecular mechanism by which cGKI inhibits InsP$_3$-dependent calcium release in smooth muscle. Subsequent work must aim to elucidate the role of InsP$_3$R in this process more precisely.

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