Calmodulin Binds to and Inhibits GTP Binding of the Ras-like GTPase Kir/Gem*

(Received for publication, August 2, 1996)
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Recently, a new subfamily of Ras-related GTP-binding proteins consisting of Rad (Ras associated with diabetes), Gem (immediate early gene expressed in mitogen-stimulated T-cells), and Kir (tyrosine kinase-inducible Ras-like) was discovered. The C terminus of these proteins contains an extension of approximately 30 amino acids not present in other members of the Ras family and which exhibits all the hallmarks typical for calmodulin (CaM)-binding domains. A peptide corresponding to the putative CaM-binding domain of the Kir/Gem protein was synthesized, and its affinity for CaM was determined by fluorescence spectrometry. Titration of dansyl-CaM with the Kir/Gem peptide gave an affinity constant of 1 nM. Furthermore, a single point mutation of the peptide, W269G, abolished this high affinity interaction. Gel-shift analysis showed that the complex formation between CaM and the Kir/Gem peptide is strictly calcium-dependent. We also demonstrate with a newly developed [32P]CaM overlay technique that full-length Kir/Gem and Rad proteins bind CaM in a Ca2+-dependent fashion. The binding of CaM to glutathione S-transferase-Kir and GST-Gem inhibited the binding of GTP to Kir/Gem significantly. These results suggest the existence of a direct link between Ca2+/CaM and growth factor signal transduction pathways at the level of small Ras-like GTPases.

Calmodulin (CaM) acts as the intracellular calcium sensor that translates the Ca2+ signal into a variety of cellular processes. Biochemical characterization of the interaction of CaM with its targets has defined its role as an activator of multiple Ca2+/CaM-dependent enzymes important in various cellular functions including growth and cell division (1). One of the most crucial roles of Ca2+/CaM appears to be the activation of immediate early genes. Ca2+/CaM is able to stimulate a number of transcription factors such as c-Fos, c-Jun, the cyclic AMP-response element-binding protein, and the serum response factor (2, 3). Except for the known action of CaM and CaM kinase II on cyclic AMP-response element-binding protein activation by phosphorylation and the involvement of CaM and calcineurin in NF-AT-dependent gene activation (4), a direct link between Ca2+-CaM and growth factor-dependent signal transduction pathways at the level of upstream signaling molecules such as Ras, inositol 1,4,5-trisphosphate signaling molecules, and mitogen-activated protein kinases has not yet been established molecularly.

Recently, a new subfamily of Ras-related GTP-binding proteins named Rad (5), Gem (6), and Kir (7) was discovered. Rad has a 61% amino acid sequence identity to Gem (6) and Kir (7), respectively, whereas the latter 2 proteins are 98% identical to each other. The 29-kDa Rad protein is highly expressed in skeletal muscle, lung, and heart of humans. It binds GTP and has low intrinsic GTPase activity which is greatly enhanced by a GTPase-activating protein (GAP) activity present in various tissues and cell lines (8). Rad has recently been shown to interact with skeletal muscle β-tropomyosin and the cytoskeleton of muscle cells in a guanine nucleotide-dependent manner (9), suggesting that Rad may be involved in skeletal muscle motor function and cytoskeletal organization.

Gem, a 35-kDa GTP-binding protein was cloned from mitogen-induced human peripheral blood T cells (6). The protein was found to be phosphorylated on tyrosine residues and localized to the cytosolic face of the plasma membrane. Deregulated Gem expression prevented proliferation of normal and transformed 3T3 cells, suggesting that Gem is a regulatory protein, possibly participating in receptor-mediated signal transduction at the plasma membrane.

Kir has been found to be expressed in cells transformed by ABL tyrosine kinase oncogenes (7). The Kir gene encodes a protein of 33 kDa that exhibits guanine nucleotide-binding activity. Kir was cloned by differential screening of genes present in fully malignant versus growth factor-independent cell lines expressing wild-type or mutant forms of BCR/ABL. Kir expression in BCR/ABL and v-ABL transformed B-cells renders these cells highly tumorigenic and metastatic, indicating that Kir could be involved in processes of invasion or metastasis in mammalian cells (7, 10).

Interestingly, unlike most members of the Ras superfamily, Kir/Gem and Rad do not possess the lipid modification motifs CAAX, CXC, or CC (11). However, the C terminus of the Kir/Gem and Rad proteins contains an approximately 30-amino acid extension not present in other members of the Ras family. In this study, we show that a synthetic peptide corresponding to the C terminus of the Kir/Gem protein is able to bind CaM with high affinity. Replacement of a single residue, W269G, in the peptide abolished this high affinity interaction indicating that tryptophan 269 is important for complex formation. Furthermore, binding of CaM to GST-Kir/GST-Gem inhibits GTP binding significantly.

EXPERIMENTAL PROCEDURES

Materials—The wild type Kir/Gem peptide (WT) and the mutant peptide (M1) were synthesized by manual solid phase peptide synthesis on 2-chlorotrityl resin. Fmoc-protected amino acids were activated with 2-(1H-benzotriazole/N,N,N,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole/N,N-diisopropylethylamine (1:1:2) in 2-(1H-benzotriazole/1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole/N,N-diisopropylethylamine (1:1:2) in 2-(1H-benzotriazole/1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole/N,N-diisopropylethylamine (1:1:2) in

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 41, Issue of October 11, pp. 25067–25070, 1996
Printed in U.S.A.
1-methyl-2-pyrrolidone/dimethylformamide/dichloromethane (1:1:1). The side-chain protecting groups for the fMoc amino acids were as follows: N-o-(2,2,5,7,8-pentamethylchroman-6-sulfonyl) for arginine, tri-tyl for asparagine, tertiary butylxycarbonyl for lysine and tryptophan, and tert-butyl for serine.

The WT KirGem (264/265–288/289) peptide (KARRFWGKVAKNNMAFKLKLSKS) corresponds to residues 264 to 288 of the reported Kir sequence or residues 265 to 289 of the reported Gem sequence, respectively (6, 7). In the mutant peptide (M1), the tryptophan residue 269 was replaced by a glycine residue. The peptides were purified by preparative reverse-phase high performance liquid chromatography on a Nucleosil C8 column (30 nm, 10 μm, 250 × 55 mm Machery and Nagel) as described by Anagli et al. (12). The purity of the peptides was confirmed by amino acid analysis and mass spectrometry. Dansylcalmodulin was purchased from Sigma. GST-Rad, GST-Gem, and GST-Kir were kindly provided by Drs. R. C. Kahn, K. Kelly, and O. Witte, respectively.

**Fluorescence Measurements**—Fluorescence measurements were performed with a SPEX Fluorolog 1680 (Metuchen, NJ) double-wavelength spectrophotometer connected to a SpectraAcq Control Module and run on a Datamax version 1.03 software (Jobin Yvon/Spex) as described by Kelly and O. Witte, respectively.

**Generation of the GST-CaM construct**—GST-CaM was overexpressed and purified from E. coli with a histidine tag. The purity was determined by SDS-PAGE and mass spectrometry. The GST-CaM construct was then used in the binding assays.

**RESULTS AND DISCUSSION**

To investigate the possibility of a direct link between calcium and the Ras-like GTPases, we used a CaM-binding assay. The CaM-binding assay was performed according to Ref. 14. Slab gels of 12% acrylamide, 4 μm urea, 0.375 M Tris-HCl, pH 8.8, and 0.1 mM CaCl₂ (A) or 2 mM EGTA (B) were run at a constant current of 25 mA. All lanes contain 300 pmol of CaM. Lane 1, calmodulin alone; lane 2, plus 300 pmol of peptide; lane 3, plus 600 pmol of peptide; lane 4, plus 900 pmol of peptide.

We first asked whether the putative CaM-binding domain of Kir/Gem indeed is able to bind CaM. Therefore, the peptide KARRFWGKVAKNNMAFKLKLSKS corresponding to residues 264 to 288 of the reported Kir sequence or residues 265 to 289 of the reported Gem gene product was synthesized. We have introduced a single amino acid substitution, glycine for tryptophan at position 269 of the wild type Kir peptide (M1) to obtain a negative control peptide. Nondenaturing gel electrophoresis in the presence of 4 μm urea revealed that the Kir/Gem peptide bound to CaM and induced a shift of the complex in the presence of 0.1 mM CaCl₂ (Fig. 2A). Addition of 2 mM EGTA abolished completely complex formation indicating that the interaction of Kir/Gem with CaM was Ca²⁺-dependent (Fig. 2B). Maximum shift was observed when the CαM peptide ratio was 1:1. A molar excess of peptide did not result in the appearance of new bands, suggesting that CaM interacts with the Kir/Gem peptide with a 1:1 stoichiometry. No significant shift was observed when the mutant peptide M1 was analyzed (data not shown).

The interaction between the Kir/Gem peptide and CaM was also studied using fluorescence spectroscopy. As shown in Fig. 3A, titration of dansylcalmodulin with the Kir/Gem peptide saturated in the nanomolar concentration range in the presence of 0.1 mM CaCl₂. The maximum in the emission spectrum of the binding peptide underwent a shift to lower wavelength and an increase in intensity upon complex formation with dansylcalmodulin indicating protein/peptide interaction. No increase in intensity and shift of the spectrum was observed in control experiments (peptide alone plus calcium, dansylcalmodulin alone plus calcium, data not shown). The CaM-peptide interaction was Ca²⁺-dependent since addition of 1 mM EGTA reversed both the shift and intensity of the spectrum (Fig. 3B). Interestingly, the mutant Kir/Gem peptide (M1) is completely inactive in CaM binding under the same experimental conditions. No shift to lower wavelength and no increase in fluorescence intensity could be observed (Fig. 3A). From this result we can conclude that the tryptophan residue at position 269 of the Kir/Gem peptide is important for the high affinity interaction of CaM with Kir/Gem and second that the tryptophan residue at position 269 directly participates in complex formation. Similar results were also obtained with other mutant peptides (data not shown). The affinity constant for the formation of the complex between CaM and the Kir/Gem peptide was determined from titration experiments with dansylcalmodulin. The Kir/Gem
peptide bound CaM with high affinity (1 nM) (Fig. 4). The plot of relative fluorescence versus the molar ratio of Kir/Gem to dansylcalmodulin as shown in Fig. 5 indicates Ca²⁺ dependence of the interaction and a stoichiometry of 1:1. This is in agreement with the results obtained using urea gel-shift analysis (Fig. 2). The formation of the CaM/peptide complex was also monitored at different free calcium concentrations (Fig. 6). Half-maximal binding was obtained with less than 10 µM free calcium. In summary, using both methods, gel-shift analysis and fluorescence spectrometry, we have shown that the Kir/Gem peptide binds CaM in a strictly Ca²⁺-dependent fashion and with a stoichiometry of 1:1. Furthermore, the binding of the Kir/Gem peptide to CaM is abolished when the single tryptophan residue in the Kir/Gem peptide is replaced by a glycine residue.

Additionally, we addressed the question whether the full size Kir/Gem and Rad proteins retain the ability to bind CaM. A rapid alternative method for detecting CaM-binding proteins with a ³²P-labeled CaM probe generated as a GST-fusion protein was used to detect GST-Kir/Gem and GST-Rad in an overlay assay. Fig. 7 demonstrates that full-length Kir/Gem and Rad proteins are easily detected by the ³²P-labeled GST-CaM probe. As a control, GST alone does not bind to either Kir/Gem or Rad (data not shown). Furthermore, Fig. 7 shows that a large amount of the three proteins is associated with the pellet fraction of bacterial extracts. In addition to GST-Gem (calculated molecular mass of 60 kDa), a higher molecular mass species (over 150 kDa), which also gives a strong signal, is present in the total lysate and the pellet fraction. Interestingly this band is not present in GST-Kir and GST-Rad bacterial lysates, suggesting a Gem-specific effect.

Since Kir, Gem, and Rad have been described as GTP-binding proteins (5–8), we investigated whether CaM binding affects GTP binding to Kir/Gem. As can be seen from Fig. 8, Kir/Gem binds GTP less efficiently in the presence of CaM. We can observe an inhibition of GTP binding of about 40%. This result would suggest that CaM alone is not sufficient to suppress GTP binding, but could affect the level of bound nucleotides through an unknown mechanism. No GAP or GRF for Kir/Gem and Rad have been described, but CaM could play a regulatory role in conjunction with effector molecules such as GAP's or GRF's specific for Kir/Gem.

The C terminus of the Gem gene product was shown to be important for intracellular localization (6). A C-terminal truncated form of Gem redistributes from the plasma membrane to the nucleus, whereas N-terminal truncated Gem resides mostly at the plasma membrane. CaM binding to the C terminus of
Kir/Gem and Rad proteins might modulate or regulate their intracellular localization. CaM might therefore be involved in a novel mechanism regulating the attachment to or release from the plasma membrane of these Ras-like GTPases.

Evidence for a direct link between Ca\(^{2+}\) and the Ras signal transduction pathway was reported for the neuronal exchange factor RasGRF as a signal mediator and CaM target by Farnsworth et al. (19). Activation is mediated in a Ca\(^{2+}\)-dependent fashion by CaM binding to RasGRF. Recently, a novel GTPase-activating protein, called IQGAP, has been reported (20, 21). Interestingly, IQGAP also binds CaM (21). The existence of a CaM-binding domain in Ras-like GTPases such as Kir/Gem (this study) and in effector molecules of Ras and Ras-like G-proteins such as RasGRF and IQGAP is intriguing. One can speculate that CaM-binding motifs (similarly to the ones in Ras-like G-proteins such as RasGRF and IQGAP is intriguing. One can speculate that CaM-binding motifs (similarly to the ones in Ras-like G-proteins such as RasGRF and IQGAP) may represent yet another important module regulating protein-protein interactions in signal transduction pathways. The physiological relevance of such interactions, however, has yet to be investigated.

That Ras-like proteins of the Kir/Gem and Rad type could play an important role in growth-related signaling has recently been demonstrated by the finding that Kir functions upstream of the ste20 kinase in activating mitogen-activated protein kinase cascades in yeast (10). Since the function of Kir to induce the pseudohyphae formation phenotype in yeast is abolished when Kir lacks the C-terminal domain Ca\(^{2+}\)-CaM may be involved in the regulation of biological functions of small Ras-related proteins.

Acknowledgments—We thank Drs. R. C. Kahn, K. Kelly, and O. Witte for providing us with GST-Rad, GST-Gem, and GST-Kir, respectively.

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Fig. 6. Calcium dose-response curve. Percentage of relative fluorescence is plotted as a function of the free Ca\(^{2+}\) concentration in the incubation buffer. CaCl\(_2\) was added to yield the free Ca\(^{2+}\) concentrations indicated on the abscissa, calculated with the help of a computer program derived from Ref. 22.

Fig. 7. GTP overlay assay. GST-Kir, GST-Gem, and GST-Rad were run on 12% SDS-PAGE, blotted onto NC filters, and incubated with a \(^{32}\)P-labeled GST-CaM probe. T, total lysate; S, supernatant of cell lysate; P, pellet.

Fig. 8. Guanine nucleotide-binding assay. GTP binding to GST-Kir/GST-Gem was determined using a nitrocellulose filtration assay. GST-Kir/GST-Gem (20 pmol) was incubated in an exchange buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mg/ml bovine serum albumin, 1 mM MgCl\(_2\), and 5 \(\mu\)Ci of [\(^{32}\)P]GTP in the presence or absence of 100 pmol CaM for the indicated times. Circles, absence of CaM; rectangles, presence of CaM. Each data point is the average of duplicate determinations.

TIME (min)