Localization of the Sites for Ca 2+ −Binding Proteins on G Protein−Coupled Receptor Kinases †

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Localization of the Sites for Ca^{2+}-Binding Proteins on G Protein-Coupled Receptor Kinases†

Konstantin Levay,‡ Daulet K. Satpaev,‡ Alexey N. Pronin,§ Jeffrey L. Benovic,§ and Vladlen Z. Slepak*,‡

Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33136, and Departments of Biochemistry & Molecular Pharmacology and Microbiology & Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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ABSTRACT: Inhibition of G protein-coupled receptor kinases (GRKs) by Ca^{2+}-binding proteins has recently emerged as a general mechanism of GRK regulation. While GRK1 (rhodopsin kinase) is inhibited by the photoreceptor-specific Ca^{2+}-binding protein recoverin, other GRKs can be inhibited by Ca^{2+}−calmodulin. To dissect the mechanism of this inhibition at the molecular level, we localized the GRK domains involved in Ca^{2+}-binding protein interaction using a series of GST−GRK fusion proteins. GRK1, GRK2, and GRK5, which represent the three known GRK subclasses, were each found to possess two distinct calmodulin-binding sites. These sites were localized to the N- and C-terminal regulatory regions within domains rich in positively charged and hydrophobic residues. In contrast, the unique N-terminally localized GRK1 site for recoverin had no clearly defined structural characteristics. Interestingly, while the recoverin and calmodulin-binding sites in GRK1 do not overlap, recoverin−GRK1 interaction is inhibited by calmodulin, most likely via an allosteric mechanism. Further analysis of the individual calmodulin sites in GRK5 suggests that the C-terminal site plays the major role in GRK5−calmodulin interaction. While specific mutation within the N-terminal site had no effect on calmodulin-mediated inhibition of GRK5 activity, deletion of the C-terminal site attenuated the effect of calmodulin on GRK5, and the simultaneous mutation of both sites rendered the enzyme calmodulin-insensitive. These studies provide new insight into the mechanism of Ca^{2+}-dependent regulation of GRKs.

G protein-coupled receptor kinases (GRKs)[1] are a family of serine/threonine protein kinases that phosphorylate activated G protein-coupled receptors (*1*, *2*). GRK-phosphorylated receptors then associate with arrestin proteins, leading to uncoupling of receptor and G protein and in some cases initiating the process of receptor internalization (*3*−*7*). In mammals, six distinct members of the GRK family have been identified. These 60−80 kDa soluble enzymes have significant structural homology in their centrally localized catalytic domains, while their N- and C-terminal regulatory domains are more divergent. Primarily on the basis of differences within these regulatory domains, the GRKs have been divided into three subclasses. The first subclass contains GRK1 (rhodopsin kinase), which is found only in photoreceptor cells and the pineal gland (*8*). The second class consists of the ubiquitous GRK2 and GRK3 (β-adrenergic receptor kinases) (*9*), while the third class contains GRK4 and GRK5, which are expressed in several cell types, and the ubiquitous GRK6 (*10*−*12*). The C-terminal regulatory domains of GRKs differ in length and are particularly divergent among the GRK subclasses. These regions are implicated in GRK translocation to the membrane, a key prerequisite for receptor phosphorylation. The diversity of the C-terminal domains apparently reflects the variety of mechanisms of membrane attachment employed by GRKs. GRK1 is post-translationally farnesylated (*13*). GRK2 and GRK3 associate with membranes through their C-terminal pleckstrin homology domains in a process facilitated by G protein $\beta\gamma$-subunits released upon G protein activation (*14*−*17*). GRK5 binds to the membrane via a stretch of positively charged residues, while GRK4 and GRK6 are palmitoylated within the C-terminal region (*1*).

While GRKs employ distinct mechanisms for membrane association, their inhibition by EF-hand Ca^{2+}-binding proteins appears to be common to all three subclasses. GRK1 was the first member for which Ca^{2+}-mediated inhibition was discovered (*18*). GRK1 inhibition appears to be due to the direct binding of recoverin, a 23 kDa photoreceptor-specific Ca^{2+}-binding protein (*19*, *20*). More recently, it has been found that all other GRKs are inhibited by the Ca^{2+}-binding protein calmodulin (*12*, *21*−*23*). While most of these studies analyzed the effect of Ca^{2+}−calmodulin on GRK-mediated phosphorylation of rhodopsin in vitro, it was also demonstrated that increased intracellular Ca^{2+} levels result in

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* Corresponding author.

‡ University of Miami School of Medicine.

§ Thomas Jefferson University.

[1] Abbreviations: GST, glutathione *S*-transferase; GRK, G protein-coupled receptor kinase; SPR, surface plasmon resonance; ROS, rod outer segments; PIP_2, phosphatidylinositol 4,5-bisphosphate.

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decreased m2 muscarinic acetylcholine receptor desensitization in CHO cells (*22*).

Since Ca^{2+}−calmodulin has multiple targets, a potential approach to studying the role of GRK regulation by Ca^{2+} in vivo is the use of GRK mutants deficient in calmodulin binding. However, the current information about the structure−function aspects of GRK interaction with Ca^{2+}-binding proteins is not sufficient to design such mutants, although we have previously identified a calmodulin-binding site in the N-terminal domain of GRK5 (*23*). Here, we further localize the sites for Ca^{2+}-binding proteins on GRK1, GRK2, and GRK5. Surprisingly, all three GRKs contain two separate sites for calmodulin binding. These sites are localized within the N- and C-terminal regulatory domains, and contain short stretches of positively charged and hydrophobic residues which are characteristic of calmodulin-binding domains in other proteins (*24*). In GRK5, the C-terminal site appears to play the major role in the inhibition of GRK5, while the simultaneous mutation of both binding sites results in a kinase that is completely insensitive to calmodulin.

MATERIALS AND METHODS

Materials. GRK1 was a gift from J. Hurley (University of Washington, Seattle, WA). The enzyme was purified from Sf9 cells by affinity chromatography on immobilized *Escherichia coli*-expressed recoverin (*20*). Bovine GRK2 and human GRK5 were expressed and purified from Sf9 cells as described previously (*10*, *25*). Calmodulin was from Sigma or Calbiochem, and troponin C was a gift from J. D. Potter (University of Miami, Miami, FL).

Expression and Purification of Glutathione S-Transferase (GST) Fusion Proteins. The various GST−GRK fusion constructs were obtained by PCR-mediated cloning of GRK fragments into pGEX vectors (Pharmacia). The proteins were expressed in *E. coli* strain BL21 and purified on glutathione−agarose. Bacteria were induced with 0.1 mM IPTG for 2 h at 30 °C, harvested, washed with ice-cold buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA, and lysed in the presence of 100 μg/mL lysozyme and protease inhibitors. Lysis was completed using a French Press, and the lysate was then clarified by centrifugation at 20000*g* for 15 min at 4 °C. Supernatants were incubated with glutathione−agarose for 30 min on ice and washed three times with ice-cold PBS and once with storage buffer [20 mM HEPES (pH 7.5), 100 mM NaCl, and 1 mM $MgCl_2$]. Bound GST−GRK proteins were then eluted in the same buffer containing 10 mM reduced glutathione. The fusion proteins were transferred to the desired buffer by desalting on a Sephadex G15 column. The purity of the proteins was 80−95% as determined by Coomassie Blue staining. Protein concentrations were determined by dye binding assay (Bio-Rad) using bovine serum albumin as a standard.

Analysis of Protein−Protein Binding. Calmodulin−Sepharose (Pharmacia) was used for testing GRK1−calmodulin interaction by affinity chromatography. In a typical experiment, 20 μL of beads was washed and equilibrated in loading buffer [20 mM HEPES (pH 7.4), 100 mM NaCl, and 1 mM $MgCl_2$ containing either 1 mM Ca^{2+} or 5 mM EGTA]. GRK1 was loaded batchwise for 30 min at room temperature in a total volume of 40 μL. The suspension was then transferred to a microcolumn, and unbound material (20 μL) was collected. After the beads were washed with loading buffer (2 × 20 μL), GRK1 was eluted with the same buffer containing 20 mM EGTA (2 × 100 μL). Aliquots of collected fractions were analyzed by SDS−PAGE and visualized with Coomassie Blue staining.

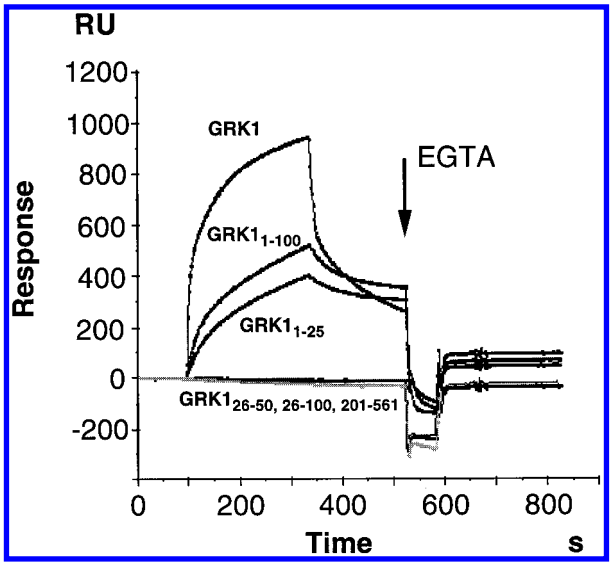

FIGURE 1: Recoverin binds to the N terminus of GRK1. Overlay plot of the real-time binding data recorded upon interaction of immobilized recoverin with recombinant GRK1 or GST−GRK1 fusion proteins. Recoverin was covalently attached to the surface of the BIACORE sensor chip via a cysteine residue as described in Materials and Methods. The traces (sensograms) reflect the amount of protein complex formed at the surface over time. Each sensogram consists of an association phase during the sample flow and a dissociation phase reflecting the complex decay when the GRK is removed from the microflow cell. GRK1 (250 nM) and GST−GRK fusion proteins (1 μM) were injected at a flow rate of 10 μL/min in the presence of 500 nM Ca^{2+}. The arrow indicates the injection of 25 mM EGTA which caused rapid dissociation of the GRK1−calmodulin complex. Results with $GRK1_{1-50}$ were nearly identical to those with $GRK1_{1-25}$ and $GRK1_{1-100}$, while GST alone or various GST−GRK2 and GST−GRK5 fusion proteins did not bind to recoverin.

Surface plasmon resonance (SPR) analysis of protein−protein interactions was carried out using the BIAcore 2000 instrument (BIACORE, Inc.). Recoverin was covalently linked to carboxymethylated dextran on the surface of a CM5 sensor chip (BIACORE) via its unique cysteine residue as described previously (*26*, *27*). As a control for nonspecific binding, a surface without recoverin (immobilized cysteine or untreated surface) was tested in all experiments. Calmodulin has a strong net negative charge and thus cannot be directly coupled covalently to the CM5 chip due to electrostatic repulsion from the surface. Therefore, we first biotinylated calmodulin in solution using iodacetamide−LC-biotin (Pierce) and then coupled it to streptavidin-coated sensor chips SA (BIACORE) as previously described (*23*). Samples of full-length GRKs and GST−GRK fusion proteins were prepared in a running buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 0.01 mg/mL BSA, and 0.005% surfactant P-20 (BIACORE). The concentration of Ca^{2+} was 500 μM unless specified otherwise. To analyze the Ca^{2+} concentration dependence of GRK−calmodulin interaction, GRKs or GST−GRK fusion proteins were made in solutions without Ca^{2+} or EGTA and then diluted 40−

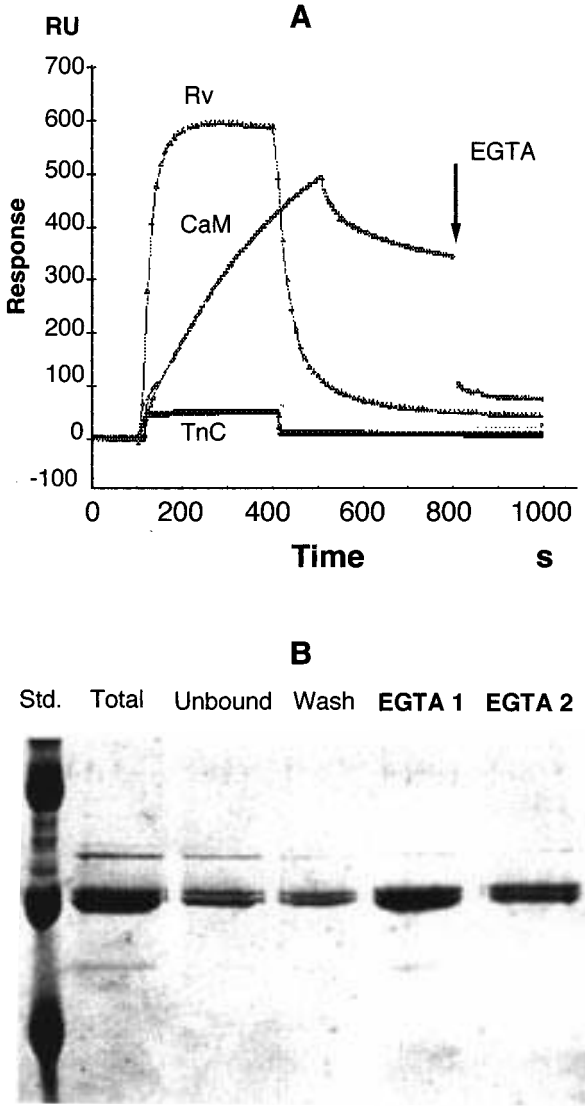

FIGURE 2: Binding of GRK1 to calmodulin. (A) Overlay plot of sensograms recorded on injection of 1 μM GRK1 over surfaces containing the immobilized Ca^{2+}-binding proteins recoverin (Rv), calmodulin (CaM), and troponin C (TnC). The arrow indicates the injection of 25 mM EGTA which caused rapid dissociation of the GRK1−calmodulin complex. While no binding of GRK1 to troponin C (a Ca^{2+}-binding protein from muscle cells closely related to calmodulin) was detected, troponin I (a TnC-binding protein) bound to the TnC surface in a Ca^{2+} concentration-dependent manner (not shown). (B) Binding of GRK1 and GST−GRK fusion proteins to calmodulin−Sepharose. GRK1 (10 μg) was incubated with 20 μL of calmodulin−Sepharose in a buffer containing 1 mM free Ca^{2+}, and the beads were washed and then eluted with 25 mM EGTA as described in Materials and Methods. Aliquots of each fraction [material loaded onto the matrix (total), flow-through (unbound), wash, and two EGTA eluates] were analyzed by SDS−PAGE.

50-fold in buffers containing free Ca^{2+} at a predetermined concentration (Molecular Probes).

The equilibrium binding constant, K_D, was deduced from a series of injections of GRK at different concentrations using two methods: (1) the half-maximal concentration required for saturation and (2) the kinetics of binding from the off and on rates ($K_D = k_d/k_a$). The determination of the rate constants was done using BIAEvaluation 2.1 software (BIACORE). There was some variability in the K_D values depending on the batch of GRK, particularly GRK1, apparently due to variation in the content of active protein due to

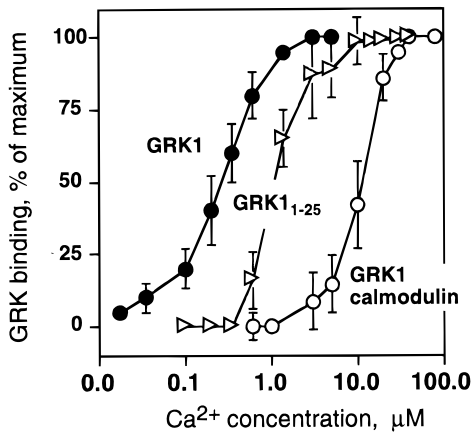

FIGURE 3: Effect of Ca^{2+} on GRK1 binding to recoverin and calmodulin. Recoverin (● and tilted triangle) or calmodulin (○) was immobilized on the chip, and solutions of 0.5 μM GRK1 (● and ○) or 1 μM GST−GRK1$_{1-25}$ (tilted triangle) at different concentrations of free Ca^{2+} were injected across the sensor surface. GRK1 and GST−GRK1$_{1-25}$ were prepared in buffers containing no EGTA or divalent cations and then diluted 50-fold in solutions with buffered Ca^{2+} concentrations (Molecular Probes). Binding was measured at the equilibrium achieved for each of the tested Ca^{2+} concentrations and is expressed as a percentage of the maximum response reached at a saturating Ca^{2+} concentration. Data were from two independent experiments with GRK1$_{1-25}$ and from five or six experiments with GRK1.

instability and aggregation. However, with the same preparation of GRK, the difference between independent experiments was negligible. The same method of kinetic analysis was used to assess interactions with the GST−GRK fusion proteins.

GRK Activity. Transfection of COS-1 cells and measurement of GRK activity were carried out as described earlier (*23*). GRK expression plasmids were constructed by cloning the coding sequences of wild-type or mutated (generated by PCR) human GRK5 in the vector pcDNA3. COS-1 cells were grown to ~80−90% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were transfected with 4 μg of plasmid DNA per 60 mm cell culture dish using LipofectAMINE (Gibco BRL). Fourty-eight hours after transfection, cells were harvested by scraping into 0.3 mL of ice-cold 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 250 mM NaCl, and 1% Triton X-100 with protease inhibitors. Cellular extracts were prepared by centrifugation for 7 min at 100000*g* and 4° C. GRKs were then partially purified by chromatography on SP Sepharose as described previously (*28*). For activity assays, partially purified GRKs were incubated in ambient light for 6 min at 30 °C with rod outer segment membranes (100 pmol of rhodopsin) in 20 μL of 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM [γ-^{32}P]ATP (1000 cpm/pmol) in the presence of calmodulin at the indicated concentration. The samples were subjected to electrophoresis on a 10% SDS−polyacrylamide gel, and the gels were stained with Coomassie Blue, dried, and subjected to autoradiography and the ^{32}P-labeled proteins excised and counted. The effect of GST−GRK fusion proteins on calmodulin-stimulated GRK5 autophosphorylation was measured using the GRK5 mutant GRK5-DD (Ser484 and Thr485 both mutated to Asp) as previously described (*23*). Four picomoles of GRK5-DD was labeled in the presence

Table 1: Binding of GRKs and GST−GRK Fusion Proteins to Calmodulin and Recoverin[a]

GRK or fragment	K_D (M)	k_d (s^{-1})	k_a (M^{-1} s^{-1})	$K_{1/2}$ for Ca^{2+} (μM)[b]
GRK1				
recoverin	$(1.0 \pm 0.1) \times 10^{-6}$	$(1.0 \pm 0.2) \times 10^{-1}$	$(1.0 \pm 0.1) \times 10^{5}$	0.3 ± 0.1
calmodulin	$(1.0 \pm 0.1) \times 10^{-7}$	$(1.0 \pm 0.1) \times 10^{-3}$	$(1.1 \pm 0.1) \times 10^{4}$	3.0 ± 0.5
$GRK1_{1-100}$				
recoverin	$(1.0 \pm 0.2) \times 10^{-7}$	$(1.0 \pm 0.1) \times 10^{-3}$	$(1.1 \pm 0.2) \times 10^{4}$	3.0 ± 1.0
calmodulin	$(2.0 \pm 0.2) \times 10^{-8}$	$(1.4 \pm 0.2) \times 10^{-3}$	$(5.5 \pm 1.0) \times 10^{4}$	10.0 ± 2.0
$GRK1_{1-50}$				
recoverin	$(1.0 \pm 0.2) \times 10^{-7}$	$(1.0 \pm 0.5) \times 10^{-3}$	$(1.0 \pm 0.5) \times 10^{4}$	
calmodulin	$(5.0 \pm 0.2) \times 10^{-7}$	$(1.5 \pm 0.4) \times 10^{-3}$	$(3.5 \pm 1.0) \times 10^{3}$	
$GRK1_{1-25}$				
recoverin	$(1.0 \pm 0.1) \times 10^{-7}$	$(1.0 \pm 0.1) \times 10^{-3}$	$(1.0 \pm 0.2) \times 10^{4}$	3.0 ± 1.0
$GRK1_{26-100}$				
calmodulin	$(2.0 \pm 0.3) \times 10^{-7}$	$(5.0 \pm 0.4) \times 10^{-3}$	$(2.5 \pm 0.4) \times 10^{4}$	
$GRK1_{26-75}$				
calmodulin	$(9.0 \pm 1.0) \times 10^{-7}$	$(3.0 \pm 0.4) \times 10^{-3}$	$(1.2 \pm 0.3) \times 10^{4}$	
$GRK1_{26-50}$				
calmodulin	$(4.0 \pm 1.0) \times 10^{-7}$	$(1.0 \pm 0.1) \times 10^{-3}$	$(3.0 \pm 0.3) \times 10^{3}$	
$GRK1_{201-561}$	$(4.0 \pm 0.2) \times 10^{-8}$	$(1.0 \pm 0.4) \times 10^{-3}$	$(2.0 \pm 0.6) \times 1\ 0^{4}$	
$GRK1_{341-561}$	$(5.5 \pm 0.6) \times 10^{-8}$	$(2.0 \pm 0.4) \times 10^{-3}$	$(3.0 \pm 0.5) \times 10^{4}$	
$GRK1_{341-400}$	$(2.0 \pm 0.4) \times 10^{-7}$	$(1.2 \pm 0.2) \times 10^{-3}$	$(1.0 \pm 0.4) \times 10^{4}$	
GRK2	$(4.0 \pm 0.2) \times 10^{-8}$	$(4.0 \pm 0.1) \times 10^{-4}$	$(1.0 \pm 0.5) \times 10^{4}$	
$GRK2_{1-147}$	$(1.2 \pm 0.2) \times 10^{-7}$	$(2.0 \pm 0.4) \times 10^{-3}$	$(2.0 \pm 0.1) \times 10^{4}$	
$GRK2_{1-88}$	$(4.0 \pm 0.2) \times 10^{-8}$	$(3.0 \pm 0.4) \times 10^{-3}$	$(8.0 \pm 2.0) \times 10^{4}$	
$GRK2_{466-689}$	$(3.0 \pm 0.2) \times 10^{-7}$	$(3.0 \pm 0.4) \times 10^{-3}$	$(3.0 \pm 0.1) \times 10^{4}$	
GRK5	$(8.0 \pm 3.0) \times 10^{-9}$	$(2.0 \pm 0.1) \times 10^{-3}$	$(2.5 \pm 0.1) \times 10^{5}$	1.3 ± 0.2
$GRK5_{1-200}$	$(1.7 \pm 0.3) \times 10^{-8}$			
$GRK5_{1-200}/A^{30}Q^{31}$	$(6.0 \pm 1.8) \times 10^{-7}$	$(2.0 \pm 0.4) \times 10^{-3}$	$(1.0 \pm 0.2) \times 10^{4}$	
$GRK5_{20-39}$	$(2.0 \pm 0.1) \times 10^{-7}$	$(3.0 \pm 0.4) \times 10^{-2}$	$(5.0 \pm 0.4) \times 10^{4}$	
$GRK5_{489-590}$	$(2.0 \pm 0.2) \times 10^{-8}$	$(1.0 \pm 0.4) \times 10^{-3}$	$(5.0 \pm 0.1) \times 10^{4}$	0.4 ± 0.2

[a] The affinity and kinetics of protein−protein interactions were studied using the BIAcore instrument. Sensograms were recorded upon consecutive injections of GRK at increasing concentrations over immobilized Ca^{2+}-binding proteins at saturating Ca^{2+} concentrations. The equilibrium binding constants (K_D) are based on the half-maximal concentration of the analyte required for saturation. The rate constants (k_d and k_a) were derived from analysis of the dissociation and association phases of the sensograms, respectively, using BIAEvaluation 2.1 (BIACORE) software. Since the rate constants and the K_D presented in the table were determined using different methods, in some cases the K_D values are slightly different from the K_D calculated using the k_d/k_a ratio. [b] GRKs or the GST−GRK fusion proteins were injected at several concentrations of free Ca^{2+} across a sensor surface containing either immobilized recoverin or calmodulin. The $K_{1/2}$ for Ca^{2+} was determined as the half-maximal concentration of free Ca^{2+} required for optimal binding at a fixed analyte concentration. The concentration of analytes was typically in the range of $0.25-2.0 \times K_D$. All results presented in the table are determined on the basis of at least two independent experiments.

of 0.7 or 2 μM GST−GRK fusion protein with or without 0.1 μM calmodulin. None of the fusion proteins affected the basal (i.e., in the absence of calmodulin) autophosphorylation of GRK5-DD.

RESULTS

Localization of the Recoverin-Binding Site on GRK1. Direct Ca^{2+}-dependent interaction of recoverin with GRK1 has been previously demonstrated by affinity chromatography and by SPR (*20*, *26*). To localize the site of recoverin interaction on GRK1, we examined the binding of a series of GST−GRK1 fusion proteins to immobilized myristoylated recoverin (Figure 1). Only the fusion proteins possessing N-terminal sequences of GRK1 readily bound to recoverin in the presence of Ca^{2+}, while constructs containing C-terminal portions of GRK1 as well as sequences from other GRKs did not bind. The shortest fragment capable of interaction contained the 25 N-terminal amino acids of GRK1 ($GRK1_{1-25}$). Binding of the N-terminal fragments was Ca^{2+}-dependent (Figure 3) and was completely blocked by recoverin delivered in solution (not shown).

The binding affinity (K_D) and on- and off-rate constants (k_a and k_d) determined for GRK1 and the GST−GRK1 fusion protein binding to recoverin are summarized in Table 1. Surprisingly, due to a slower dissociation rate, the GRK1 fragments bound to recoverin ~10-fold stronger than full-length GRK1. However, this was true only at saturating Ca^{2+} concentrations, since the half-maximal concentration of Ca^{2+} required for the binding of GRK1 fragments was significantly higher than for intact GRK1 (e.g., 3 μM for GRK_{1-100} vs 0.3 μM for GRK1). This suggests that interaction with GRK1 increases the affinity of recoverin for Ca^{2+}. As expected, ATP, which significantly decreases recoverin−GRK1 interaction due to GRK1 autophosphorylation (*26*), did not have any effect on the GST−GRK1 fusion protein binding to recoverin (not shown).

GRK1 Binding to Calmodulin. Since previous studies have demonstrated that calmodulin has no effect on GRK1 activity (*8*, *23*, *29*), we did not expect that it would bind to the kinase. However, our studies revealed a robust interaction between the two proteins as assessed by either SPR (Figure 2A) or affinity chromatography on calmodulin−agarose (Figure 2B). In contrast, denatured GRK1 did not bind to calmodulin (not shown). In addition, GRK1 also did not bind to immobilized troponin C, a Ca^{2+}-binding protein from muscle cells that is closely related to calmodulin (*30*, *31*). Thus, the GRK1−calmodulin interaction appears to be specific.

The kinetic parameters of GRK1−calmodulin binding are shown in Table 1 along with the characteristics of GRK2, GRK5, and the various GST−GRK fusion proteins. Calm-

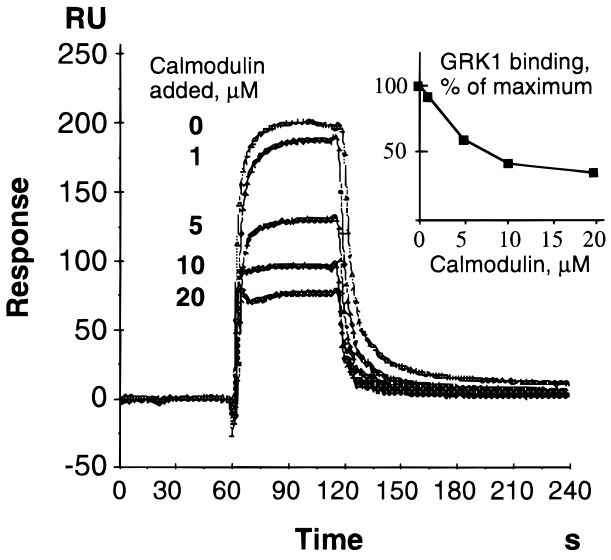

FIGURE 4: Calmodulin inhibition of GRK1 binding to recoverin. Recoverin was immobilized and 1 μM GRK1 injected in the presence of 0.1 mM Ca^{2+} and calmodulin at the indicated concentration. The overlaid sensograms show the decrease in the level of GRK1 binding to recoverin at the higher concentrations of calmodulin. The inset shows the effect of calmodulin concentration (*X*-axis) on GRK1−recoverin binding (*Y*-axis) expressed as a percentage of binding in the absence of calmodulin (100%). The data are representative of three independent experiments.

odulin binds to GRK1 with a K_D of 100 nM, compared with the K_Ds of 40 and 8 nM for calmodulin binding to GRK2 and GRK5, respectively. Surprisingly, GRK1 binds to calmodulin ~10-fold stronger than to recoverin, a specific GRK1 inhibitor. The difference in binding affinity for recoverin and calmodulin is due to the markedly slower dissociation rate of the GRK1−calmodulin complex ($k_d = 10^{-3}$ s^{-1}) compared to the rapid dissociation of GRK1 and recoverin ($k_d = 10^{-1}$ s^{-1}). In contrast, the association rate of GRK1 binding to calmodulin ($k_a = 10^4$ M^{-1} s^{-1}) is 10 times slower than that of GRK1 binding to recoverin ($k_a = 10^5$ M^{-1} s^{-1}).

As expected, GRK1 interaction with calmodulin was Ca^{2+} concentration-dependent (Figure 3). Measurements of steady-state GRK1 binding to calmodulin at different concentrations of free Ca^{2+} revealed a $K_{1/2}$ of 3 μM Ca^{2+}, similar to the $K_{1/2}$ for GRK5 interaction with calmodulin but ~10-fold higher than the 0.3 μM value for the $K_{1/2}$ for GRK1 binding to recoverin (Table 1). In addition, the Ca^{2+} concentration dependence of GRK1 binding to calmodulin was also highly cooperative (Hill coefficient of 2.1). Moreover, the binding of full-length GRKs to calmodulin required significantly lower Ca^{2+} concentrations than binding of the GST−GRK fusion proteins (Table 1), suggesting that GRK interaction increases the affinity of calmodulin for Ca^{2+}.

Calmodulin Inhibits GRK1 Binding to Recoverin. To test whether calmodulin and recoverin bind to a common site on GRK1, we assessed whether they competed for GRK1 interaction. GRK1 interaction with immobilized recoverin was significantly inhibited by co-injection of calmodulin (Figure 4). In contrast, GRK1 interaction with immobilized calmodulin was unaffected by recoverin co-injection (Figure 5A). Control studies demonstrated that calmodulin co-injection was able to effectively inhibit GRK1 binding to immobilized calmodulin as expected (Figure 5B), while

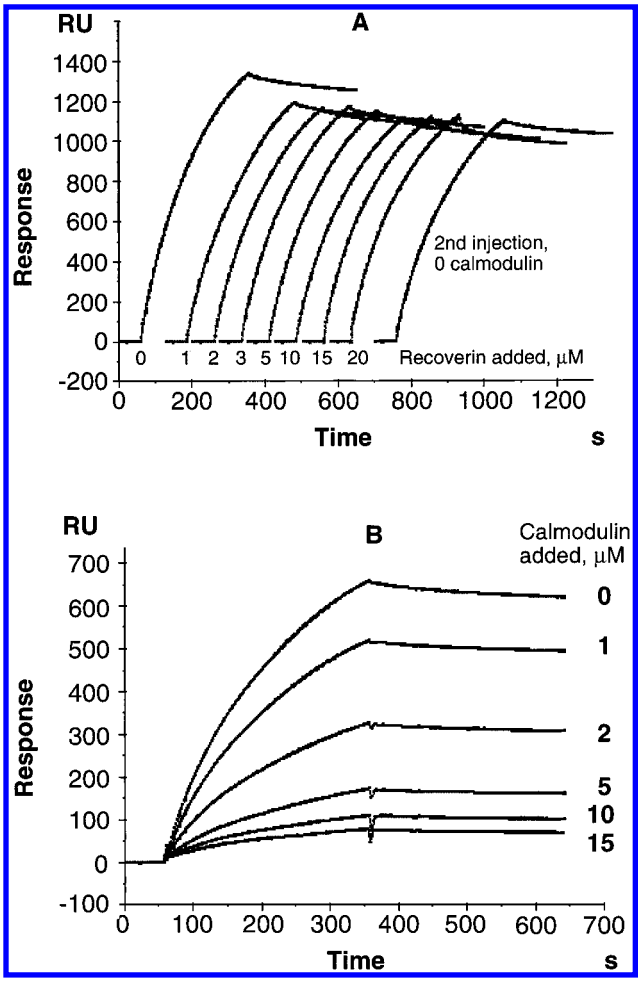

FIGURE 5: Recoverin does not inhibit GRK1 binding to calmodulin. Calmodulin was immobilized and 1 μM GRK1 injected in the presence of the indicated concentrations of recoverin (A) or calmodulin (B). (A) The sensograms are shifted along the time axis to avoid overlap. A second GRK1 injection made after six cycles of GRK1 binding and EGTA regeneration shows that the immobilized calmodulin retained more than 90% of its activity. The data are representative of three independent experiments. (B) GRK1 injected in the presence of free calmodulin resulted in inhibition of GRK1 binding to the surface. The data are representative of two independent experiments.

troponin C had no effect on GRK1 binding to recoverin or calmodulin (not shown). Moreover, there was no direct binding between recoverin and calmodulin (not shown). These results suggest that the ability of calmodulin to inhibit GRK1 binding to recoverin likely occurs allosterically rather than through competition for a common binding site. Since recoverin had no effect on GRK1 binding to calmodulin, we anticipated that an additional molecular event (e.g., binding at an additional site) might explain our results. Thus, we next set out to localize the calmodulin-binding site(s) on GRK1.

Localization of the Calmodulin- and Recoverin-Binding Sites on GRK1. Our analysis of the GST−GRK1 fusion protein binding to recoverin and calmodulin is summarized in Figure 6A and Table 1. A fusion protein containing the 100 N-terminal amino acids of GRK1 (GST−$GRK1_{1-100}$) is capable of binding to both calmodulin and recoverin. In contrast, GST−$GRK1_{1-25}$ binds only to recoverin, while GST−$GRK1_{26-100}$ and GST−$GRK1_{26-75}$ bind only to calmodulin. GST−$GRK1_{1-50}$ binds to both Ca^{2+}-binding proteins

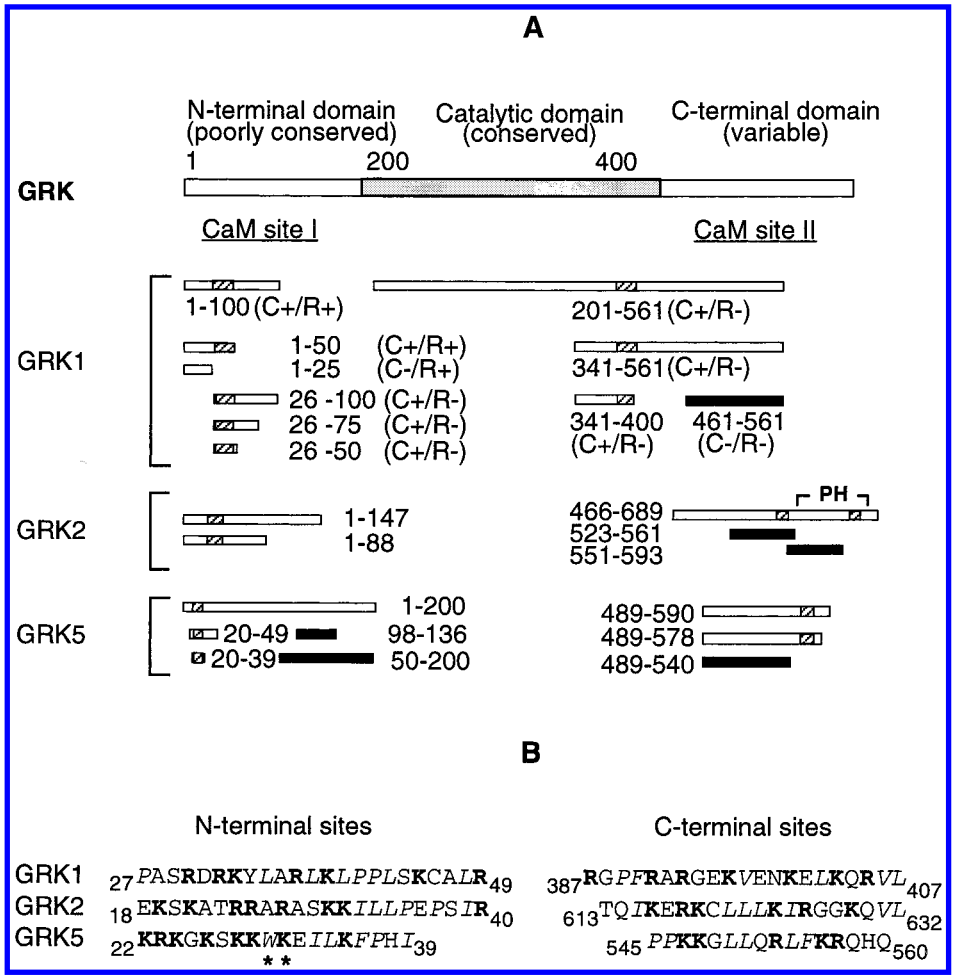

FIGURE 6: Localization of the sites for Ca^{2+}-binding proteins on GRKs. (A) Summary of the binding of GRK1, GRK2, and GRK5 fragments to recoverin (R) and calmodulin (C). The long bar (GRK) depicts the overall structure of the GRKs, while the shorter bars represent the various GST−GRK fusion proteins that were tested. The open bars represent constructs that bound a Ca^{2+}-binding protein, while the black bars did not bind. For the GRK1 fragments, a + indicates binding to calmodulin (C+) or recoverin (R+) while a − indicates no binding (C− or R−). The hatched blocks within the GRK fragments represent domains that fit the criteria of potential calmodulin-binding sites (see the text for more details). PH denotes the approximate location of the pleckstrin homology domain of GRK2. None of the GRK2 or GRK5 fragments bound to recoverin, while GST and unrelated GST fusion proteins did not bind to recoverin or calmodulin. (B) Amino acid sequences of the putative GRK calmodulin-binding sites. Positively charged residues are bold, while hydrophobic residues are italic. The asterisks indicate the positions of K^{30} and K^{31} in GRK5 that were mutated ($A^{30}Q^{31}$).

but has a reduced affinity for calmodulin compared to the $GRK1_{26-75}$ construct. To our surprise, GST fusions containing $GRK1_{201-561}$, $GRK1_{341-561}$, and $GRK1_{341-400}$ also bound to calmodulin while $GRK1_{461-561}$ did not. These results suggest the presence of a second calmodulin-binding site between residues 341 and 400 on GRK1. Thus, GRK1 has three distinct sites for interaction with Ca^{2+}-binding proteins: one for recoverin (residues 1−25) and two for calmodulin (residues 25−75 and 341−400).

Localization of Calmodulin-Binding Sites on GRK2 and GRK5. We next tested whether GRK2 and GRK5 also contained two calmodulin-binding sites. Indeed, like GRK1, GRK2 and GRK5 also have distinct calmodulin-binding sites located within the N- and C-terminal regulatory domains (Figure 6A and Table 1). The two sites are found within residues 20−39 and 540−578 in GRK5 and residues 1−88 and 593−689 in GRK2. These regions contain clusters of positively charged and hydrophobic residues (Figure 6B), a characteristic of calmodulin-binding sites found in other target proteins (*24*).

There are two major possibilities of how calmodulin might bind to GRKs. The first is that the two sites could be completely independent, resulting in one GRK interacting with two molecules of calmodulin. The second possibility would involve the two domains constituting a single interface for docking one molecule of calmodulin. In the latter case, the N- and C-terminal fragments, the putative "halves" of such a binding site, would interact with calmodulin simultaneously. To test between these possibilities, we determined whether one of the isolated calmodulin-binding domains would preclude the binding of the other domain to immobilized calmodulin using the BIAcore instrument. When calmodulin was initially saturated with the N-terminal domain (GST−$GRK5_{1-200}$) of GRK5, the C-terminal domain (GST−$GRK5_{489-590}$) was not able to bind (Figure 7). Similarly, the C-terminal domain of GRK5 precluded binding of the N-terminal domain (Figure 7). Similar results were also obtained using various combinations of the N- and C-terminal fragments of GRK1, GRK2, and GRK5. This suggests a model in which calmodulin binds to two distinct sites on GRKs with a calmodulin:GRK stoichiometry of 2:1. This is indirectly supported by the fact that the calmodulin-binding sites in many other target proteins are also relatively short domains (*24*). To further analyze the potential

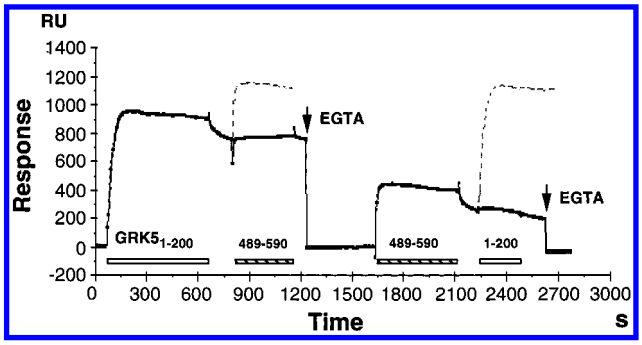

FIGURE 7: Calmodulin-binding sites are independent. GST−GRK5 fusion proteins containing residues 1−200 or 489−590 were injected across immobilized calmodulin. The surface was initially saturated with one of the fusion proteins before injection of the second. The order of the samples entering the flow cell is denoted below by the open or hatched bar. The dark sensograms show the experimental data, while the dashed line show a projection of the signal if the two GRK fragments could bind to calmodulin simultaneously. The signal at saturation is different for the two fragments because of the difference in their molecular masses.

importance of having two calmodulin-binding sites, we focused additional studies on GRK5 since it is the GRK most sensitive to calmodulin.

Interaction of Calmodulin with Separate GRK Calmodulin-Binding Domains. We initially characterized the binding of the GRK N- and C-terminal fusion proteins with respect to their kinetics and Ca^{2+} concentration dependence for interaction with calmodulin (Table 1). As might be expected, the individual domains have a lower affinity for calmodulin than the holoenzyme. Interestingly, $GRK5_{1-200}$ bound at Ca^{2+} concentrations higher than intact GRK5, while $GRK5_{489-590}$ had a reduced requirement for Ca^{2+} (Figure 8). This demonstrates that like GRK1, GRK5 increases the affinity of calmodulin for Ca^{2+} primarily through the C-terminal calmodulin-binding site. Indeed, an increased affinity of calmodulin for Ca^{2+} has been demonstrated to occur upon interaction with several other target proteins and peptides (*32*−*34*). The differential effects of Ca^{2+} on calmodulin binding to the two sites suggest that they might be engaged at different intracellular Ca^{2+} concentrations and thus play a distinct role in GRK regulation.

Role of Calmodulin Binding in GRK5 Regulation. To dissect the role of the individual calmodulin-binding sites in GRK5, we made point mutations and deletions within these domains. We first tested the effect of substitution of two residues, Trp30 and Lys31, within the N-terminal GRK5 fusion protein by making the construct $GRK5_{1-200}/A^{30}Q^{31}$. This mutation resulted in an ~30-fold decrease in the affinity for calmodulin compared to that of wild-type $GRK5_{1-200}$ (Table 1). When tested for its ability to bind calmodulin and inhibit calmodulin-stimulated autophosphorylation of GRK5, $GRK5_{1-200}/A^{30}Q^{31}$ was essentially inactive compared to wild-type $GRK5_{1-200}$ (Figure 9C). We also generated several mutations within full-length GRK5. Deletion of residues 20−39 resulted in complete loss of catalytic activity, most likely because of protein misfolding (data not shown). In contrast, GRK5 containing the W30A and K31Q mutations ($GRK5/A^{30}Q^{31}$) was active when expressed in COS-1 cells and phosphorylated rhodopsin with an efficiency similar to that of wild-type GRK5. Moreover, both GRK5 and $GRK5/A^{30}Q^{31}$ were inhibited by calmodulin with the same efficacy (Figure 9A,B). This suggests that the N-terminal calmodulin-binding domain is not critical for mediating GRK inhibition by calmodulin.

We also tested the role of calmodulin binding to the C-terminal domain of GRK5. Deletion of up to 50 C-terminal residues of GRK5 (i.e., $GRK5_{1-562}$, $GRK5_{1-551}$, and $GRK5_{1-540}$) had modest effects on the catalytic activity.[2] However, while $GRK5_{1-562}$ was inhibited by calmodulin with the same IC_{50} as wild-type GRK5 (not shown), $GRK5_{1-551}$ (and $GRK5_{1-540}$) had an ~3.5-fold reduced sensitivity to calmodulin (Figure 9A,B). This suggests that residues 552−561 play a critical role in calmodulin binding within the C-terminal domain of GRK5. To test whether the residual sensitivity of $GRK5_{1-551}$ is due to calmodulin interaction at the N-terminal site, we introduced the $A^{30}Q^{31}$ substitution into $GRK5_{1-551}$. The resulting product, $GRK5_{1-551}/A^{30}Q^{31}$, was completely insensitive to calmodulin (Figure 9A,B). Thus, the C-terminal domain (residues 552−561) is important for calmodulin-promoted inhibition of GRK5, while the N-terminal domain (residues 20−39) may also have a role in GRK5−calmodulin interaction.

DISCUSSION

Recoverin-Binding Site on GRK1. The recoverin-binding domain appears to be localized within the 25 N-terminal residues of GRK1. This region does not contain any characteristic secondary structure or homology to other proteins. Since previous studies have shown that the N-terminal region of GRK1 (residues 17−34) is likely involved in recognition of activated rhodopsin (*35*), we hypothesize that recoverin inhibits phosphorylation by directly competing with rhodopsin for binding to GRK1. Moreover, since the N-terminal domain also includes a site of autophosphorylation, Ser21 (*36*), it is possible that the inhibition of recoverin binding that occurs upon GRK1 autophosphorylation (*26*) is due to modification of this particular site.

The GST fusion proteins containing $GRK1_{1-25}$, $GRK1_{1-50}$, and $GRK1_{1-100}$ bound to recoverin with an association rate about 10 times slower than that of intact GRK1. Surprisingly, the dissociation of these proteins was ~100-fold slower, which means that the fragments had a higher apparent affinity for recoverin compared to GRK1. The dissociation rate did not depend on the surface density of recoverin, the salt or detergent concentration, or the flow rate, and the complex was completely Ca^{2+} concentration-dependent. Since $GRK1_{1-50}$ and $GRK1_{1-100}$ actually bound to calmodulin weaker than native GRK1, it seems unlikely that the increased stability of recoverin's complex with the N-terminal GRK1 constructs is due to aggregation of the GST fusions or some other artifact. Perhaps the on and off rates of recoverin binding to intact GRK1 are increased due to interaction of another region of GRK1 with recoverin. The reduced Ca^{2+} sensitivity of recoverin binding to the N-terminal GRK1 fusions also indicates that this region cannot fully mimic the holoenzyme. The EC_{50} for Ca^{2+} is decreased for the full-length kinase, suggesting that it can allosterically enhance recoverin−Ca^{2+} interaction. Thus, while the main site for recoverin binding is located at the N terminus of

[2] A. N. Pronin, C. V. Carman, and J. L. Benovic, manuscript submitted for publication.

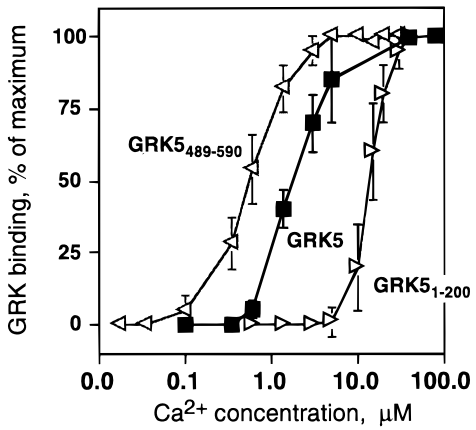

FIGURE 8: Ca^{2+} sensitivity of calmodulin interaction with GRK5 fragments. Binding of 0.5 μM GRK5 (■), GST−$GRK5_{1-200}$ (triangles pointing to the right), and GST−$GRK5_{489-590}$ (triangles pointing to the left) to immobilized calmodulin. GRK5 and the GST−GRK5 fusion proteins were prepared in solutions with buffered Ca^{2+} concentrations (Molecular Probes). The binding was measured at equilibrium achieved for each of the tested Ca^{2+} concentrations.

GRK1, additional regions may also contribute to the interaction, resulting in its characteristic rapid kinetics and high affinity for Ca^{2+}.

Role of Calmodulin−GRK Interaction. The K_D's for GRK interaction with calmodulin are as follows: GRK5 (8 nM) < GRK2 (40 nM) < GRK1 (80 nM). While this order is the same as the order for the IC_{50}'s of calmodulin-mediated inhibition of GRK activity [GRK5 (40 nM) < GRK2 (2 μM) < GRK1 (50 μM) (*22*, *23*)], the absolute values of K_D and IC_{50} differ significantly, particularly for GRK2 and GRK1. This discrepancy might be partially attributed to differences in assay conditions. For example, binding between the two proteins is measured directly in the SPR assay. In contrast, the phosphorylation reaction involves several steps, including GRK interaction with the membrane, rhodopsin, ATP, and ADP. The actual binding between GRK and calmodulin may not be the limiting step in this chain of molecular events. Furthermore, some artifacts could alter the experimentally determined IC_{50} and K_D values. For example, sequestration of calmodulin by unknown targets in the ROS membranes might reduce its effective dose, particularly at low concentrations, thereby increasing the apparent IC_{50}. In contrast, the SPR assay could decrease the K_D's due to the 1:2 stoichiometry of the GRK−calmodulin complex (see below) which could result in a slower dissociation.

The assay conditions do not explain, however, why the disagreement between K_D and IC_{50} is different among the three GRKs. While the 8 nM K_D and 40 nM IC_{50} for GRK5 deviate 5-fold, this variance is 50-fold for GRK2 and ~100-fold for GRK1. It should also be noted that the K_D of GRK1−recoverin binding determined by SPR correlates very well with the IC_{50} for recoverin-mediated GRK1 inhibition (*20*, *37*−*39*). One additional explanation for the discrepancy between the values of K_D and IC_{50} for GRK−calmodulin interaction is that other molecules might reduce binding and thus more calmodulin would be required to saturate GRK in the presence of other substrates or lipid membrane. Future experiments using plasmon resonance on a recently developed phospholipid-covered surface (*40*, *41*) will be needed to test whether the membrane and receptor have a direct effect on GRK−calmodulin binding. Our findings also suggest that GRK−calmodulin binding could have an additional function which varies among the GRK subtypes. The high potency of calmodulin toward GRK5 suggests that GRK5 inhibition might be the main physiologic role of this interaction. Since the efficacy of calmodulin in inhibiting GRK2 is lower, the significance of this interaction is less clear. However, it is possible that calmodulin could be more effective at inhibiting GRK2-mediated phosphorylation of a physiologic receptor substrate.

Since calmodulin is a very poor inhibitor of GRK1 (*8*, *23*, *29*), the potential role of calmodulin binding to GRK1 is most puzzling. Calmodulin inhibits rhodopsin phosphorylation by GRK1 with an IC_{50} of 50 μM (data not shown), ~10-fold higher than its estimated concentration in photoreceptors (*42*). By comparison, the IC_{50} for GRK1 inhibition by recoverin is ~1 μM (*20*, *38*, *39*). However, our experiments demonstrate the specificity of GRK1−calmodulin binding in vitro. (1) Troponin C, a protein closely related to calmodulin, did not bind to GRK1. (2) GRK1−calmodulin binding is Ca^{2+} concentration-sensitive. (3) Free calmodulin inhibits GRK1 binding to immobilized recoverin. (4) The affinity of GRK1−calmodulin binding is sufficient for this interaction to occur in vivo. This suggests that the function of GRK1−calmodulin binding might be different from that of the inhibition of rhodopsin phosphorylation.

One potential role of calmodulin interaction with GRK1 could be in regulating post-translational modification, similar to its role in GRK5 autophosphorylation (*23*). Another possibility is that calmodulin might not change the extent of rhodopsin phosphorylation, but rather the specificity of phosphorylation (i.e., which sites are modified). Phosphorylation of rhodopsin in vitro occurs at as many as eight serine and threonine residues. In vivo, however, phosphorylation appears to be more tightly controlled. Studies on live retinas using mass spectral analysis of rhodopsin-derived phosphopeptides reveal that labeling occurs primarily at one (*43*) or, at most, two to three residues (J. Hurley, personal communication). Importantly, the actual phosphorylation sites are dependent on the timing of illumination (*43*). Since light controls Ca^{2+} levels in photoreceptors (*19*, *44*, *45*), this might be explained by the action of a Ca^{2+}-binding protein capable of GRK1 interaction (e.g., calmodulin). Indeed, it has been shown that rhodopsin phosphorylated at different sites has an altered affinity for arrestin and for its ability to stimulate G proteins (*5*).

We still cannot rule out the possibility that calmodulin interaction with GRK1 (and GRK2) has lost its physiological significance during evolution of the GRK family. The structural variance of the GRKs and different gene structure suggest that they evolved by convergence rather than divergence (*2*). Indeed, some regulatory mechanisms, such as membrane binding and autophosphorylation, are different among the GRKs. In contrast, the two sites for calmodulin are present in all GRK subclasses, and it seems unlikely that they were retained or even independently acquired during evolution without some functional significance.

GRKs Have Two Calmodulin-Binding Sites. The N-terminal GRK calmodulin-binding sites are located similar distances from the N terminus in GRK1, -2, and -5 and display significant homology. The location of these sites in GRK1 and GRK2 was suggested previously on the basis of

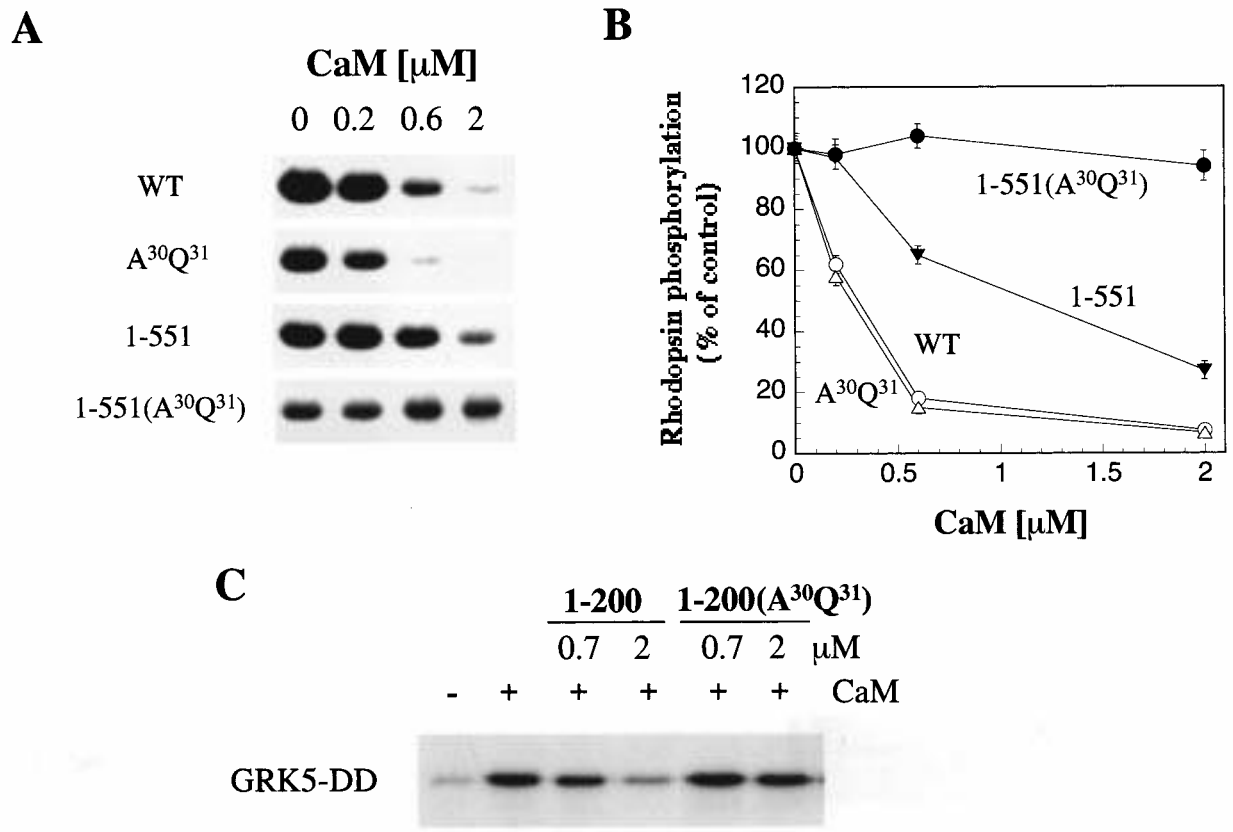

FIGURE 9: Functional role of the calmodulin-binding sites on GRK5. (A) COS-1 cells were transiently transfected with the vector pcDNA3 containing the cDNA for wild-type GRK5 (WT), GRK5/$A^{30}Q^{31}$, $GRK5_{1-551}$, and $GRK5_{1-551}/A^{30}Q^{31}$. Protein extracts from the cells were enriched in GRKs (see Materials and Methods) and incubated with ROS membranes in the presence of [γ-^{32}P]ATP and calmodulin (CaM) at the indicated concentrations. Labeled proteins were separated on a 10% SDS−polyacrylamide gel and visualized by autoradiography. (B) The amount of ^{32}P incorporation was determined by excising and counting the radioactive bands. The activity of wild-type GRK5 (△), GRK5/$A^{30}Q^{31}$ (○), $GRK5_{1-551}$ (▼), or $GRK5_{1-551}/A^{30}Q^{31}$ (●) in the presence of calmodulin is expressed as a percentage of rhodopsin phosphorylation in the absence of calmodulin. (C) Effect of GST−GRK5 fusion proteins on calmodulin-stimulated GRK5 autophosphorylation. GRK5-DD (0.2 μM) was autophosphorylated in the absence or presence of 0.1 μM calmodulin with or without the indicated concentrations of wild-type or $A^{30}Q^{31}$ GST−$GRK5_{1-200}$ fusion proteins. Proteins were separated on a 10% SDS−polyacrylamide gel and visualized by autoradiography.

structural similarity with the N-terminal domain of GRK5 (*23*). The prediction was based upon homology with calmodulin binding sites in other target proteins which generally consist of ~20 residue domains characterized by a net positive charge, a medium hydrophilicity, and a moderate to high helical hydrophobic moment (*24*, *46*). While sequence analysis suggested the existence of putative calmodulin-binding sites in the C terminus of GRKs, their activity and precise localization lacked experimental support. The C terminus of GRK2, for instance, contains two potential calmodulin-binding sites. Experiments with GST−GRK fusion proteins (Figure 6) allowed us to localize the C-terminal calmodulin-binding sites to a single region in each of the investigated GRKs. In GRK1, as in many other calmodulin targets, this site (residues 375−407) is within the kinase catalytic domain. In contrast, in GRK2 and GRK5, calmodulin binds within the C-terminal regulatory domain (residues 614−632 and 545−564, respectively). The variance in position of these sites relative to the catalytic domain suggests that the C-terminal sites evolved independently and may play distinct roles in different GRKs.

We found that the simultaneous mutation of both calmodulin-binding sites results in a GRK that is Ca^{2+}−calmodulin concentration-insensitive. While mutation of the N-terminal site alone did not change the IC_{50} for calmodulin, deletion of the C-terminal site decreased calmodulin sensitivity of the kinase. This suggests that the C-terminal site plays a major role in GRK5 inhibition. The N-terminal site may increase GRK5 sensitivity to calmodulin and expand the range of Ca^{2+} concentrations at which GRK5 remains responsive to intracellular Ca^{2+}.

Studies of numerous calmodulin-regulated proteins reveal that calmodulin binding occurs near or within such regions as pseudosubstrate domains in protein kinases and phosphatases, phosphorylation sites, or regions of interaction with other proteins (*24*). GRK-mediated receptor phosphorylation includes several steps, such as GRK translocation to the membrane, receptor−GRK interaction, activation of the kinase, phosphate transfer, and dissociation of activated GRK. An attractive hypothesis could be that each calmodulin-binding site regulates one particular molecular event in the GRK functional cycle. Since the GRK N terminus may be involved in receptor recognition (*35*), occupation of the N-terminal site by calmodulin could inhibit GRK−receptor interaction. Calmodulin binding to the C-terminal site could then attenuate the targeting of the kinase to the membrane. Unraveling these mechanisms at the molecular level as well as studying the significance of GRK regulation by Ca^{2+} in vivo can be approached with the use the GRK mutants with an altered sensitivity to calmodulin. The identification of calmodulin binding sites reported here is an important step in designing such mutants.

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