Role for the Regulator of G-Protein Signaling Homology Domain of G Protein-Coupled Receptor Kinases 5 and 6 in β2-Adrenergic Receptor and Rhodopsin Phosphorylation

Faiza Baameur, Daniel H. Morgan, Hui Yao, Tuan M. Tran, Richard A. Hammitt, Subir Sabui, John S. McMurray, Olivier Lichtarge, and Richard B. Clark

ABSTRACT

Phosphorylation of G protein-coupled receptors (GPCRs) by GPCR kinases (GRKs) is a major mechanism of desensitization of these receptors. GPCR activation of GRKs involves an allosteric site on GRKs distinct from the catalytic site. Although recent studies have suggested an important role of the N- and C-termini and domains surrounding the kinase active site in allosteric activation, the nature of that site and the relative roles of the RH domain in particular remain unknown. Based on evolutionary trace analysis of both the RH and kinase domains of the GRK family, we identified an important cluster encompassing helices 3, 9, and 10 in the RH domain in particular. To define its function, a panel of heptad repeat domains was generated and screened by intact-cell assays of constitutive GRK phosphorylation of the β2-adrenergic receptor (β2AR), in vitro GRK phosphorylation of light-activated rhodopsin, and basal catalytic activity measured by tubulin phosphorylation and autophosphorylation. A number of double mutations within helices 3, 9, and 10 reduced phosphorylation of the β2AR and rhodopsin by 50 to 90% relative to wild-type GRK, as well as autophosphorylation and tubulin phosphorylation. Based on these results, helix 9 peptide mimetics were designed, and several were found to inhibit rhodopsin phosphorylation by GRK5 with an IC50 of ~30 μM. In summary, our studies have uncovered previously unrecognized functionally important sites in the regulator of G-protein signaling homology domain of GRK5 and -6 and identified a peptide inhibitor with potential for specific blockade of GRK-mediated phosphorylation of receptors.

To understand the mechanism underlying the activation of GRKs by the GPCRs, it is essential to identify functional sites in the GRKs involved in their activation. The serine/threonine GRK family includes seven members, GRK1–7, classified into three subfamilies on the basis of their sequence homology: the rhodopsin kinase subfamily (GRK1 and -7), activities of which are restricted to the visual system; the β2-adrenergic receptor kinase subfamily (GRK2 and -3); and the GRK4 subfamily (GRK4–6) (Krupnick and Benovic, 1998; Pitcher et al., 1998). Crystal structures have been determined for GRK2 in complex with the βγ subunits of G proteins (Lodowski et al., 2005), for GRK6 bound to Gαq subunits of G proteins (Lodowski et al., 2003, 2005, 2006; Tesmer et al., 2005), and recently for six crystal structures of rhodopsin kinase (Singh et al., 2008). These structures all seem to be in the inactive state. Thus, neither the active-state conformation of GRKs nor how they interface with GPCRs is known.

Membrane localization and activation of GRKs are complex, involving several domains within the kinase. It was shown that the GRK2 N-terminal fragment (residues 45–178) coimmunoprecipitated with metabotropic glutamate receptor 1 (Dhami et al., 2002) and that a single mutation in the β3-adrenergic receptor kinase subfamily (GRK2 and -3); and the GRK4 subfamily (GRK4–6) (Krupnick and Benovic, 1998; Pitcher et al., 1998). Crystal structures have been determined for GRK2 in complex with the βγ subunits of G proteins (Lodowski et al., 2005), for GRK6 bound to Gαq subunits of G proteins (Lodowski et al., 2003, 2005, 2006; Tesmer et al., 2005), and recently for six crystal structures of rhodopsin kinase (Singh et al., 2008). These structures all seem to be in the inactive state. Thus, neither the active-state conformation of GRKs nor how they interface with GPCRs is known.

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GRK2 (D527A) in the RH domain disrupted the GRK2-mGluR1 interaction (Dhami et al., 2004). In addition, an early study showed that the binding of an antibody to the N terminus of GRK1 blocked receptor phosphorylation (Palczewski et al., 1993). Moreover, in a yeast screen study, mutations within the GRK5 N terminus were found to impair membrane localization and block rhodopsin phosphorylation (Noble et al., 2003). In a recent extension of these studies, it was found that several N-terminal mutations of GRK2 (D3K, L4A, and D10A) caused severe impairment of activity, leading to the conclusion that the extreme N terminus is involved in an intramolecular interaction that enhances GRK2 activity (Pao et al., 2009). Other studies suggested a role for the C terminus in the recognition of active GPCRs. A proline-rich motif within the C terminus of GRK1, GRK2, and GRK5 mediates its association with light-activated rhodopsin (Gan et al., 2004). In addition, it was shown that the C-terminal amphipathic helix of both GRK5 and -6 was required for plasma membrane localization and substrate (rhodopsin, tubulin) phosphorylation (Thiyagarajan et al., 2004; Jiang et al., 2007). It was also suggested that the dimer interface of GRK6 is a likely area of protein-protein interactions (Lodowski et al., 2006), and that residues 5 to 30 of the N terminus and the C-terminal extension of the kinase domain proximal to the hinge region are possible sites for allosteric receptor binding (Singh et al., 2008). It has been found that mutations in the GRK2 kinase domain, notably V477D, impair its phosphorylation of rhodopsin and β2AR (Sterne-Marr et al., 2009). In addition, a number of mutations in the kinase small and large lobes of GRK1 (R191A/K, Y274A, V476A, and V484A) were found to block rhodopsin phosphorylation (Huang et al., 2009), and it was proposed that a cooperative interaction between the N terminus and the kinase domain was important for GPCR activation. In summary, although much has been learned from these studies, at present there has been no direct demonstration that mutant GRKs are defective in binding to GPCRs. This reflects the many difficulties associated with developing assays in these systems that resolve the complexities and potential overlap of GRK domains responsible for membrane binding, GPCR binding, and the transition to the active state.

To address the mechanism of GRK activation, we sought first to identify evolutionarily important sites of the GRK family that may be involved directly or indirectly in GPCR activation by evolutionary trace (ET). Past studies have shown that functionally important residues in proteins can be identified computationally by ET based on how well their sequence variations correlate with evolutionary divergence patterns (Lichtarge et al., 1996; Mihalek et al., 2004). Using ET analysis, we found a number of statistically significant clusters in both the RH and kinase domains that suggest evolutionarily important regions specific to the GRK subfamily relative to the RGS proteins and serine/threonine protein kinase superfamilies, respectively. We focused the present study on the RH terminal subdomain cluster involving helices 3 and 9, helix 10 proximal to the hinge region between RH and kinase domains, and the N terminus. Our mutagenesis and functional studies were performed on GRK5 and -6, because our previous work and that of others demonstrated an important role for these GRKs in β2AR GRK site phosphorylation and desensitization (Shenoy et al., 2006; Violin et al., 2006; Tran et al., 2007; Wang et al., 2008). Our findings demonstrate the importance of sites in helices 3, 9, and 10 in the RH domain in GPCR phosphorylation and suggest that they play a key role in supporting the conformational shift of the GRKs to the activate state.

Materials and Methods

Human embryonic kidney (HEK293) cells were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents are from Mediatech (Herndon, VA). Lipofectamine 2000, and TOP 10 competent cells are from Invitrogen (Carlsbad, CA). Peptide N-glycosidase F was from New England Biolabs (Ipswich, MA). Polyclonal primary antibodies to pSε-335,356, to the C-tail of the β2AR, and to GRK2, GRK5, and GRK6 are from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-c-Myc antibody was purchased from Biovest International Inc./NCCC (Minneapolis, MN). N-terminal 6 His-tagged, recombinant, full-length, human GRK5 was purchased from Millipore (Dundee, Scotland, UK). The horseradish peroxidase-conjugated secondary antibody was from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence SuperSignal reagent was purchased from Thermo Fisher Scientific (Waltham, MA), and blue X-ray film was from Phenix (Candler, NC). QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). SP-Sepharose Fast Flow was purchased from GE Health care (Chalfont St. Giles, Buckinghamshire, UK). Purified tubulin from bovine brain was purchased from Cytoskeleton (Denver, CO). Peptides 1 to 5 were purchased from Genemed Synthesis, Inc. (San Antonio, TX).

Cell Culture. HEK293 cells stably overexpressing FLAG-tagged wild-type (WT) β2AR (WT-β2AR) at 2 to 4 pmol/mg membrane were grown in 5% CO2 at 37°C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml G418. When seeding cells for experiments dishes were coated with poly-L-lysine to aid attachment. ET Analysis. To rank the relative evolutionary importance of GRK sequence residues, we applied ET analysis (Lichtarge et al., 1996; Mihalek et al., 2004) separately to the RH and kinase domains of GRK. The RH trace was done in two ways, first as part of a global alignment of 270 aligned RGS proteins, and then including only 56 aligned sequences of GRKs (GRK1–7) from different species. Likewise, the kinase domain was traced first as part of a global alignment of 463 aligned Ser/Thr kinases, and then including only 50 GRK kinase domains. Residues in the top 30th percentile rank of importance produced clusters in the structure of GRK6 (Protein Data Bank code 2aex) as measured by a clustering z-score, which indicates important sites for structure and function above a threshold of 2 (Mihalek et al., 2003). The GRK trace residues that overlapped those from the superfamily analyses of RGS and Ser/Thr kinase were considered global determinants not necessarily specific to GRK, but those that were traced uniquely among GRKs were considered specific to the latter (Lichtarge et al., 1997; Madabushi et al., 2004).

Mutagenesis of GRK5 and GRK6. The WT hGRK5 (NM_005308), hGRK6 (NM_001004106), and hGRK2 (NM_001619) cDNA plasmids were cloned into pcDNA3.1+. The cDNA plasmid of membrane tethered GRK2-PP was also cloned into pcDNA3.1+. It was constructed by adding a cDNA sequence to its C terminus that encodes GFP2tag and an extra 17-amino acid k-ras sequence (KDGGKDKKSSKTCKVIM). This peptide contains a polybasic region and a prenylation site to ensure its plasma membrane localization and block rhodopsin phosphorylation and desensitization (Shenoy et al., 2006; Violin et al., 2006). Polyclonal primary antibodies to pSε-(355,356), to the C-tail of β2AR, and to GRK2, GRK5, and GRK6 are from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-c-Myc antibody was purchased from Biovest International Inc./NCCC (Minneapolis, MN). N-terminal 6 His-tagged, recombinant, full-length, human GRK5 was purchased from Millipore (Dundee, Scotland, UK). The horseradish peroxidase-conjugated secondary antibody was from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence SuperSignal reagent was purchased from Thermo Fisher Scientific (Waltham, MA), and blue X-ray film was from Phenix (Candler, NC). QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). SP-Sepharose Fast Flow was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Purified tubulin from bovine brain was purchased from Cytoskeleton (Denver, CO). Peptides 1 to 5 were purchased from Genemed Synthesis, Inc. (San Antonio, TX).
transfected with 150 ng of WT or mutant GRK5/6 and 1.35 ng of empty vector (pcDNA3.1+) cDNA plasmids using Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Controls were transfected with empty vector only. After 48 h, cells were treated with the β2AR agonist isoproterenol (ISO; 100 nM) dissolved in the carrier 0.1 M ascorbate/1 mM thiourea pH 7 (AT) or AT alone for 2 min. The β2ARs were then solubilized as described previously (Tran et al., 2004). Samples were resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted first with anti-pS-355,356 antibody, then stripped and reprobed with anti-C-Tail antibody, and stripped again and reprobed with the anti-GRK5/6 antibodies. Results were normalized to the β2AR levels (anti-C-tail) then to GRK5/6 levels (anti-GRK5/6).

Preparation of GRK5/6 from 21K Membrane Fractions. WT-β2AR cells were grown to ~60 to 70% confluence in 100-mm dishes. Cells were transfected with 8 μg of cDNA plasmid of either vector, WT, or mutant GRK as described above. After 48 h, cells were washed with ice-cold PBS and scraped into 1 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 3 mM benzamidine), followed by homogenization. 21K membrane fractions were prepared as described previously (Tran et al., 2007) and, for solubilization, suspended in 1 ml of lysis buffer supplemented with 50 mM NaCl, 0.02% TritonX-100, and 1 mM dithiothreitol. Samples were frozen at −80°C and used directly after dilution (see below) or partially purified on SP-Sepharose columns. The levels of GRKs were quantified by reference to standard curves generated with purified GST-GRKs as described previously (Tran et al., 2007).

Purification of GRK5 on SP-Sepharose. Cell lysates were diluted 10 times, in lysis buffer supplemented with 50 mM NaCl, 0.02% TritonX-100, and 1 mM dithiothreitol, and applied to an SP-Sepharose column. The resin (250 μl) was washed six times with 2 ml of lysis buffer before eluting GRK5 with 20 mM Tris-HCl, 1 mM EDTA, 450 mM NaCl, and 0.02% Triton X-100. The partially purified GRK5 was then diluted to a final salt concentration of 50 mM NaCl and assayed for rhodopsin phosphorylation. Purification of GRK5 was assessed by Western blot analysis. No GRK other than GRK5 was detectable in this fraction, and the level of GRK5 was assessed by reference to standard curves generated with purified GST-GRK5 or 6His-GRK5 obtained from Millipore.

Phosphorylation of Rhodopsin. Urea-striped rod outer segment membranes were prepared as described previously (Wilden and Kuhn, 1982). WT and mutant GRK, solubilized from WT-β2AR 21K membrane fractions or after purification with SP-Sepharose chromatography, were diluted in lysis buffer (5–10 nM GRK5 and GRK6) and incubated with 4 μM rhodopsin in buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM MgCl2, and 100 μM [γ32P]ATP containing ~500 dpm/pmol) at 30°C in a final volume of 32 μl (Prion and Benovic, 1997). Rhodopsin was activated by illumination (475 nm) for 30 s (Ridge et al., 2006) just before incubation. Reactions were stopped after 10 min by addition of 4× SDS-sample buffer, and samples were electrophoresed on a 12% SDS-PAGE. After transfer to nitrocellulose membranes, 32P-labeled proteins were visualized by autophosphorylation, were incubated for 1 h at 30°C and stopped by addition of 40 μl of a 50% slurry of protein A-Sepharose and further incubation for 1 h. The immunoprecipitated complex was collected and washed three times with 1 ml of 20 mM HEPES, pH 8.0, 500 mM NaCl, and once with 1 ml of 20 mM HEPES, pH 8.0. Forty microliters of the final wash buffer was then added to the pellet and assayed for both autophosphorylation and tubulin phosphorylation.

Autophosphorylation. To assay autophosphorylation, 5 μl of 5× kinase buffer (100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 25 mM MgCl2, and 100 μM [γ32P]ATP containing ~5000 dpm/pmol) was added to tubes containing 20 μl of c-Myc-immunoprecipitated GRK5. Samples were incubated for 1 h at 30°C and stopped by addition of 5× SDS-sample buffer. After gel electrophoresis and transfer to nitrocellulose membranes, 32P-labeled GRK5 was visualized by autoradiography and normalized to GRK5 levels quantitated by Western blot analysis.

Tubulin. Tubulin phosphorylation was assayed as described for autophosphorylation except for the addition of 500 nM tubulin to the reaction. 32P incorporation into tubulin was measured, and results were normalized to GRK5 levels.

Computational Design of Peptide Mimetics of Helix 9. The GRK5 native helix 9 (PDPLFLQWKLWE) is predicted by AGADIR (Munoz and Serrano, 1994) to have less than 5% helical propensity when it is in solution as a monomer (the top-ranked ET residues are underlined). This suggests that this fragment by itself may not fold into the appropriate biologically relevant helix, so that it will be less active alone than when it is part of the whole structure. As an alternative, we sought to generate peptides that were more likely to natively fold into helices but that also preserved the key residues that ET predicted should be important to function. A Peptide Builder algorithm was created to design three such helical peptides. For example, in the sequence x-x-x-F166-x-x-R169-x-x-Q172-W173-x-x-L176-x, which shows the ET residues and the remaining free residues (underlined). This suggests that this fragment by itself may not fold into the appropriate biologically relevant helix, so that it will be less active alone than when it is part of the whole structure. As an alternative, we sought to generate peptides that were more likely to natively fold into helices but that also preserved the key residues that ET predicted should be important to function. A Peptide Builder algorithm was created to design three such helical peptides. For example, in the sequence x-x-x-F166-x-x-R169-x-x-Q172-W173-x-x-L176-x, which shows the ET residues and the remaining free residues (x), x was replaced by more than 500 iterations under the condition that a replacement was accepted only if helicity increased, or until a helicity of 85% helicity was reached. Peptide Builder generated a list of peptides that could be ranked by predicted helicity, hydrophobicity, or charge. From this list, three peptides (1, 2, and 3) were selected as shown in Table 1.

Synthesis of Chemically Modified Peptides. The following method was used to generate GRK5 helix 9 peptides that were chemically modified to create side-chain–to–side-chain bridges to induce helical constraints as shown in Table 1 (peptides 7 and 8). Peptides 6, 7, and 8 were acetylated on the N terminus and were synthesized as C-terminal amides. Polydimethylacrylamide-based PL-DMA resin (Varian, Inc., Palo Alto, CA) was treated overnight with neat ethylenediamine as described by Arshady et al. (1981). After thoroughly washing the resin with DMF/CHCl3, Fmoc-Rink amide linker was added in 3-fold excess, as calculated from the nominal loading of 1 mmol/g. Coupling was mediated with 3-fold excesses of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, hydroxybenzotriazole, and a 6-fold excess of N,N-diisopropylethylamine. On completion of the coupling as judged by negative ninhydrin tests, the resin was drained, washed with DMF/CHCl3, and CH2Cl2, then dried under vacuum and stored. By weight gain, the loading was 0.65 mmol/g. Peptides 6, 7, and 8 were synthesized in parallel on aliquots of 0.20 g of this resin (0.65 mmol/g, 0.13 mmol) on a multiple synthesizer (348; AAPPtech Louisville, KY) employing a 16-well reactor block. Fmoc-amino acids were added in 3-fold excess, and coupling was mediated by N,N-diisopropylcarbodiimide/hydroxybenzotriazole in 7 ml of DMF/CH2Cl2 (1:1). Note that Met165 was replaced by Nle to avoid oxidation of the sulfur group.
Results

Evolutionary Trace Analysis of GRKs. To identify functionally important residues, we performed ET analysis of the GRK subfamily by first running two separate analyses for both the RH (Fig. 1, A–D) and the kinase (Fig. 1, E–G) domains. The RGS proteins superfamily analysis included 270 protein sequences, and the Ser/Thr kinase superfamily included 463 sequences (Fig. 1, B and F, respectively). ET analysis was used to rank the evolutionary importance of each sequence residue over all of the proteins in the alignment. Thus, when applied to an entire family, the top-ranked residues that typically form clusters can be thought of as global determinants of function. In a second phase, we restricted ET analysis to the GRK subfamily only (56 sequences), thereby generating a different set of rank scores for each residue, such that the difference between the two traces allowed us to isolate GRK-specific determinants from global determinants. Our study focuses on both GRK5 and -6, and because these two kinases are >70% homologous in their amino acid sequences and show near identical homology in the crucial region of the RH domain (Loudon and Benovic, 1994), we mapped our ET results onto the GRK6 structure (Lodowski et al., 2006) because no crystal structure has yet been published for GRK5.

For the RGS protein superfamily, our analysis of the top 30% ranked ET residues showed a cluster centering on helices α4, α5, and α7 (Fig. 1B). A number of these residues in the GRK subfamily were conserved in both the GRK/RGS superfamily and kinase domains. Evolutionary Trace shows strong clustering of the top 30% ranked residues mapped onto the GRK6 crystal structure. A–D, ET results mapping the important residues onto the RH domain of GRK6 with helix 11 and the kinase domain removed. A, results of the GRK subfamily alone with a large cluster in the terminal subdomain. B, ET results for the superfamily confirm global conservation in the bundle subdomain. C, the difference ET for the RH domain. D, close up of conserved residues clustering in helices 3, 9, and 10 (red residues are subfamily-specific, whereas pink residues are conserved in both the GRK/RGS superfamily). E–G, ET results for the kinase domain. E, results of the GRK subfamily alone. F, results of the kinase superfamily; the top 30% ranked residues are shown in yellow for both the superfamily and subfamily; the green molecule represents 5’-adenylylimidodiphosphate.

Table 1

Sequences and modifications of the helix 9 peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EFDRRWRQWRELWLR</td>
<td>Modified sequence except for ET residues, designed by PB</td>
</tr>
<tr>
<td>1AA</td>
<td>Ac-EFDRRWRQWRELWLR-NH₂</td>
<td>(AA) Acetylated and amidated peptide 1</td>
</tr>
<tr>
<td>2</td>
<td>DFEERRRQWLILYR</td>
<td>Modified sequence except for ET residues, designed by PB</td>
</tr>
<tr>
<td>2AA</td>
<td>Ac-DFEERRRQWLILYR-NH₂</td>
<td>(AA) Acetylated and amidated peptide 2</td>
</tr>
<tr>
<td>3</td>
<td>EEFDRRWRQWRELWLR</td>
<td>Modified sequence except for ET residues, designed by PB</td>
</tr>
<tr>
<td>4</td>
<td>TFMPFQGKRNL</td>
<td>Unmodified GRK6 a9 (166–177)</td>
</tr>
<tr>
<td>5</td>
<td>Ac-DFEPFQGKRNL-NH₂</td>
<td>Acetylated and amidated GRK5 a9 (166–177)</td>
</tr>
<tr>
<td>6</td>
<td>Ac-SNleDFEPFQGKRNL-NH₂</td>
<td>Acetylated and amidated GRK5 a9 (164-M165Nle-178)</td>
</tr>
<tr>
<td>7</td>
<td>Ac-SNleFFDRFLQWKWLER-NH₂</td>
<td>Glu170 side chain–Lys174 side chain bridge</td>
</tr>
<tr>
<td>8</td>
<td>Ac-SNleFFDRELQWKWLER-NH₂</td>
<td>Glu167 side chain–Lys171 side chain bridge</td>
</tr>
</tbody>
</table>

PB, peptide builder.
GRK2 helix 5 are known to form a binding interface with the Goq subunit of the G protein both from mutagenesis and the crystal structure [Arg106, Asp110, Leu118, and Gln133 (α6)] (Sterne-Marr et al., 2003; Day et al., 2004). In addition to this site, the GRK subfamily analysis revealed a separate cluster of important residues (Fig. 1C). Difference analysis (Fig. 1D) reveals a conserved cluster including helices α0, α1, α3, α9, α10, and α11, presumably domains functionally important in the GRK subfamily. Results for the difference ET analysis of the kinase domain superfamily and GRK subfamily are shown in Fig. 1G. Although in the present study these residues were not further examined, ET revealed interesting features consistent with results from the mutagenesis studies outlined above (Huang et al., 2009; Sterne-Marr et al., 2009).

To enable a better visualization of the total trace results, analyses for both the RH and the kinase domains were combined and projected on the GRK6 structure (Lodowski et al., 2006) as shown in Fig. 2 and on a surface diagram (Supplemental Fig. S2). The domains unique to the GRK subfamily for the RH domain (red) and the kinase domain (yellow) are contrasted with those residues shared by the GRK subfamily with the superfamilies (pink and orange, respectively). Based on the evidence provided by the ET for the potential importance of helices α0, α3, α9, and α10, residues in these regions were mutated and analyzed for functional effects. It is noteworthy that part of this site is buried under the C terminus; however, we reasoned that this site was still close enough to the surface to be accessible to GPCRs and that the C-terminal tail as well as the N terminus might possibly undergo conformational rearrangements that expose it further.

**Mutagenesis of Top-Ranked Residues in GRK5: Effects on Constitutive Phosphorylation of the β2AR.** To test whether the RH domain sites identified by ET analysis are involved in activation of GRKs by GPCRs (the top-ranked red residues shown in Figs. 1D and 2), single and double mutations of a number of key trace residues in helices 3, 9, and 10 of GRK5 were generated by alanine substitution to avoid introducing any charged amino acids. Alanine mutations of these residues are justified because most do not exhibit an alanine in different GRKs, and if they do, this is rare and in a distant branch. Constitutive GRK site phosphorylation was then used as a screen to assess WT and mutant GRK5 activity. We have shown an important role of GRK5 in phosphorylation of S(355,356) of the β2AR using both HEK293 and COS7 cells, and that transient overexpression of GRK5 causes a strong constitutive phosphorylation of the β2AR in these cells, unlike GRK2 (Tran et al., 2004; Tran et al., 2007). Choosing this approach to evaluate GRK mutants is based on considerable evidence that overexpression of the β2AR correlates with an increasing fraction of constitutively active receptor (R*) (Samama et al., 1993; Whaley et al., 1994). The 30- to 50-fold overexpression of GRKs leads to constitutive phosphorylation of the β2AR consistent with the fact that it reflects ligand-induced phosphorylation. The advantage of this method stems from it allowing assessment of mutant activity in an intact cell setting.

To optimize the assay, we first determined the level of GRK plasmid to achieve approximately half the GRK site phosphorylation relative to 100 nM ISO stimulation. Cells were transfected with a range of cDNA plasmid levels, and a level of 150 ng/well (35 mm) was found to be optimal and was used in the constitutive assays of the mutants. A typical result demonstrating the constitutive phosphorylation of the β2AR by WT GRK2, -5, and -6 and membrane-tethered GRK2 is shown in Fig. 3. Phosphorylation of the receptor was measured by Western blotting with the anti-pS(355,356) anti-
body (Tran et al., 2004, 2007) followed by normalization to the level of receptor expressed (C-tail antibody). It can be seen that GRK5 and -6 gave similar levels of constitutive phosphorylation of the β2AR. Consistent with previous results, GRK2 gave no response above controls, although when membrane-tethered, its activity approached that of GRK5 and -6.

The results of our screen for the single and double mutations of GRK5 in the constitutive assay are shown in Fig. 4. All results from Western blots were normalized to the total receptor level (C-tail antibody) and to the level of GRK5 expressed. Single mutations of GRK5 were consistently active as the WT GRK5, with the exception of R68A, which showed double the activity relative to WT GRK5. In contrast to other, more deleterious substitutions than alanine.

![Fig. 4. Effect of GRK5 mutations on constitutive β2AR GRK site phosphorylation. WT-β2AR cells were transiently transfected with WT or mutant GRK5. After 48 h, cells were solubilized, lysates were run on SDS-PAGE, probed with anti-pS355,356, and then stripped and reprobed with anti-C-tail, and with anti-GRK5. The data were normalized to the total receptor levels and GRK5 levels. Several double mutations show significant decrease in GRK site phosphorylation (***, p < 0.001; *, p < 0.05 by one-way ANOVA). The data are means ± S.E.M. of at least four experiments performed in duplicate. Representative Western blots are shown below the graph.](image-url)
pression of the mutants is equivalent to that of the WT, most of the mutants showed low recovery in the purification process. Therefore, we performed an assay of the WT and mutant GRK5 by use of either direct solubilization of transfected cells or by generation of a 21K pellet followed by extraction of the GRK5 as outlined under Materials and Methods. Because identical activity of the WT GRK5 was obtained from either assay with no detectable background from endogenous GRKs, we used the solubilized 21K pellet fraction. In addition, because GRK expression tended to be more variable in 100-mm dishes, we first monitored levels of expression by comparison to GST-tagged GRK5 as the standard as previously reported (Tran et al., 2007), and then adjusted levels such that approximately equivalent amounts of GRK constructs were used in the assay. The results shown in Fig. 5 demonstrate that the key GRK5 double mutants that were inactive in the constitutive phosphorylation assay displayed much reduced activity (92–98%) in their phosphorylation of Rhodopsin, whereas the WT GRK5 as well as a double mutant (P61A-Q69A) with WT levels of activity in the constitutive Rho*, whereas the WT GRK5 as well as a double mutant such that approximately equivalent amounts of GRK were solubilized (21K pellet fraction). In addition, because GRK expression tended to be more variable in 100-mm dishes, we first monitored levels of expression by comparison to GST-tagged GRK5 as the standard as previously reported (Tran et al., 2007), and then adjusted levels such that approximately equivalent amounts of GRK constructs were used in the assay. The results shown in Fig. 5 demonstrate that the key GRK5 double mutants that were inactive in the constitutive phosphorylation assay displayed much reduced activity (92–98%) in their phosphorylation of Rhodopsin, whereas the WT GRK5 as well as a double mutant (P61A-Q69A) with WT levels of activity in the constitutive β2AR phosphorylation showed robust activity. It is noteworthy that no activity from endogenous GRKs was detected after transfection with the control pcDNA3.1 plasmid. Furthermore, we detected no constitutive phosphorylation of dark-adapted rhodopsin in our assay either with increased Rho (as high as 32 μM) or up to 40-fold higher GRK5 levels (data not shown), consistent with cis-retinal acting as an inverse agonist (Gether and Kobilka, 1998).

**WT and Mutant GRK5 Autophosphorylation and Tubulin Phosphorylation.** To further investigate GRK5 mutant effects on kinase catalytic activity, we monitored basal activity using two approaches: autophosphorylation and phosphorylation of tubulin, a nonreceptor substrate for GRKs (Carman et al., 1998). c-Myc-tagged WT and several double mutants of GRK5 (F166A-P61A, F166A-L66A, F166A-W173A, Q172A-L176A) solubilized from 21K membrane fractions were immunoprecipitated and assayed for autophosphorylation. Mutants defective in constitutive β2AR and rhodopsin phosphorylation also showed reduced autophosphorylation (>95% reduction relative to WT GRK5) (Fig. 6A). Immunoprecipitated GRK5 was also examined for phosphorylation of tubulin. The defective mutants were also impaired in tubulin phosphorylation; activities were reduced nearly to control levels (Fig. 6B). The c-Myc WT GRK5 consistently showed slower mobility on SDS gels relative to the mutants. This might be attributable to autophosphorylation because it is lacking in the mutants. Collectively, these results demonstrate that the key GRK5 mutations inhibit basal kinase activity similar to their effect on receptor-stimulated activities.

**Effects of WT and Mutant GRK6 on Constitutive β2AR and Light-Activated Rhodopsin Phosphorylation.** Evolutionary trace of the GRK family demonstrated that all of the GRKs shared a core of evolutionarily important residues in helices 3, 9, and 10; therefore the above observation in GRK5 should carry over to other members of the family. Because studies by us and others indicate that GRK6 plays a role in β2AR phosphorylation, we generated and tested several single and double mutations of GRK6 that our studies with GRK5 showed to be important as well as some novel combinations. Their activity was determined in both the constitutive assay after transient expression in WT-β2AR cells, and the in vitro assay with rhodopsin using 21K membrane fractions expressing GRK6 constructs. Our findings shown in Fig. 7 demonstrate that key double mutations localized to helices 3 and 9 showed a 75 to 90% reduction in constitutive β2AR phosphorylation. Expression of most of the GRK6 mutants was reduced by ~20 to 40% relative to WT GRK6, and two mutants, Q172A and Q172A-L176A failed to be expressed and could not be assayed. Mutations that reduced GRK6 activity in the constitutive assay also showed >95% reduced rhodopsin phosphorylation. It is noteworthy that mutant L66A-R69A, expression of which did not affect significantly β2AR constitutive phosphorylation, showed ~75% reduced rhodopsin phosphorylation (Fig. 8). These mutations also include combinations of residues in helices 3 and 9 or within helix 9 alone: Y166A-L66A, L66A-Q172A, Y166A-Q172A, Y166A-L176A, and Q172A-W173A. Thus, although our mutagenesis of GRK6 was not as extensive as of GRK5, overall the results provide further support for the importance of these helices in GPCR activation of GRKs.

**Peptide Inhibition of GRK5 Phosphorylation of Light-Activated Rhodopsin.** Based on our ET analysis and experimental observations of marked diminutions of select GRK5 and -6 double mutants of helices 3 and 9 on β2AR and rhodopsin phosphorylation, it seemed possible that a peptide mimic of one of these helices that specifically conserved the key evolutionary residues might inhibit GRK activity. To address this question, we designed a series of peptide mimetics of helix 9 and tested whether they would show inhibition in the in vitro rhodopsin assay. Peptides shown in Table 1 were synthesized as follows: 1) the native sequence of GRK5 (peptides 5 and 6), and GRK6 (peptide 4); 2) peptides designed by the Peptide Builder such that important ET residues were kept unchanged, whereas others were modified to optimize helicity and solubility (1,
1AA, 2, 2AA, and 3), and 3) peptides chemically modified to
lock helix 9 in an α helix (7 and 8). Peptide (100 μM)
inhibition of partially purified GRK5 phosphorylation of
rhodopsin was then examined as shown in Fig. 9A. Of the
three Peptide Builder-designed peptides, 1 and 1AA
showed ~73 and ~86% inhibition, respectively. Peptides 2
and 3 had much reduced inhibitory activity. For the native
helix 9 peptide of GRK5, peptide 5 (residues 166–177) or
peptide 6 (164–178), in which Met165 is replaced by Nle to
avoid oxidation of the sulfur group, we found inhibitions of
~66 and ~82%, respectively. The native GRK6 helix 9 (pep-
tide 4), which differs in only two residues from that of GRK5,
showed inhibition of rhodopsin phosphorylation similar to that
of GRK5, demonstrating that both the native peptides were
active. For the chemically locked peptides 7 and 8, inhibitions
were ~85 and ~51%, respectively, indicating that helicity is
important. None of the peptides significantly inhibited GRK5
phosphorylation of Rho* at 10 μM; however, at 30 μM, peptides
1AA, 4, and 6 showed significant inhibition (45, 40, and 63%
respectively) (Fig. 9B). These findings demonstrate that the
native helix 9 peptides, locked peptides, and a peptide present-
ing the key residues on one face of the helix all inhibited phos-
phorylation. To further investigate the kinetics of peptide 6
inhibition, GRK5 activity was examined over varied concentra-
tions of rhodopsin. The results shown in Fig. 9C suggest that
the interaction of peptide with rhodopsin was complex, altering
both the apparent $K_a$ as well as the $V_{max}$, although the pre-
dominant effect was on the $V_{max}$. We also examined the speci-
city of peptides 4 and 6 for GRK5 versus GRK6. As shown in
Fig. 9D, although both peptides inhibited GRK5 phosphoryla-
tion of rhodopsin, only peptide 4 (derived from GRK6) was
effective against GRK6.

**Discussion**

The RH and kinase domain residues of GRKs evolve under
unique evolutionary selection pressure in the GRK subfamily
relative to that of the AGC kinase superfamily and, until
recently, little was known about the role conserved RH domains might play in the concerted conformational change to the active state triggered by activated GPCRs. In the present work, we initiated a difference ET study of the GRK subfamily relative to the RGS and kinase superfamilies based on the crystal structures of GRKs determined by Tesmer’s group (Lodowski et al., 2005, 2006; Singh et al., 2008). This revealed a number of sequence positions that were uniquely evolutionarily important to the GRK subfamily. Focusing on a conserved cluster within helices 3, 9, and 10 within the RH evolutionarily important to the GRK subfamily. Focusing on a conserved cluster within helices 3, 9, and 10 within the RH domain, we found numerous double mutations that significantly reduced GRK5 catalytic activity both in intact cell assays of β2AR phosphorylation and in cell-free assays of light-induced rhodopsin phosphorylation. Some of these mutants showed reduced basal activity as measured by both GRK5 autophosphorylation and tubulin phosphorylation. Several key combinations were in helix 9 alone. Other double mutations reducing activity involved either helices 3 and 10, (L66A-E514A), 9 and 10 (F166A-E514A), or loop αa-α1 in combination with helix 3 (L66A-H38A and L66A-P37A).

**Fig. 8.** Effect of GRK6 mutations on Rho* phosphorylation. WT and mutant GRK6 were expressed in WT-β2AR cells, solubilized from the 21K membrane fractions (~5 nM GRK6), and assayed as described in the legend to Fig. 5. The percentage activity of mutant GRK6 compared with WT GRK6 was calculated and normalized to GRK6 expression levels. The data are means ± S.E.M. of three experiments performed in duplicate (+++, p < 0.001 by one-way ANOVA). Inset, representative autoradiogram and GRK6 expression levels.

**Fig. 9.** Peptide inhibition of GRK phosphorylation of Rho*. Illuminated rhodopsin was incubated with SP-Sepharose-purified GRK5 in the absence (Ctrl) or presence of the peptides listed in Table 1. Bsl (basal phosphorylation) refers to samples incubated with nonilluminated rhodopsin and in the absence of peptide. A, peptides were used at 100 μM. Data shown are means ± S.E.M. for at least three experiments performed in duplicate (+++, p < 0.001; ++, p < 0.01; *, p < 0.05 by one-way ANOVA). A representative autoradiogram is shown below the graph. B, peptides 1AA, 4, 6, and 7 at 30 μM were used at 10, 30, and 100 μM. Data shown are means ± S.E.M. for three experiments performed in duplicate, except for peptide 4 (two experiments in duplicate at 10 and 30 μM). P values for peptides 1AA, 4, 6, and 7 at 30 μM were <0.01, <0.05, <0.001, and >0.05, respectively by one-way ANOVA. C, increasing concentrations of Rho* (0–25 μM) were incubated with purified 6His-GRK5 (4.6 nM) in the absence (Ctrl) or presence of peptide 6 (100 μM) for 10 min at 30 °C. The experiment shown is representative of four similar experiments each performed in duplicate. Kinetic parameters for the control and peptide treated, respectively, were as follows: Vₘₐₓ = 4.0 ± 1.1 and 1.0 ± 0.1 nmol/mg/min and Kₘₐₓ = 11.7 ± 6.9 and 21.1 ± 4.0 μM, respectively. An autoradiogram is shown below the graph. D, peptides 4 and 6 (100 μM) were incubated with Rho* (4 μM) and either GRK5 (open bars) or GRK 6 (filled bars) for 10 min at 30 °C. Data shown are the means ± S.E.M. for three experiments performed in duplicate and normalized to percentage of control. A representative autoradiogram is shown below the graph.
Identical and closely related mutations of GRK6 also showed reduced activity in both the β2AR and the rhodopsin assays, demonstrating that the effects of these mutations are not limited to GRK5 and could be observed with other subfamilies of the GRKs. It is noteworthy that our findings with both assays were generally in good agreement, although somewhat stronger inhibitions of our mutations were obtained in the rhodopsin assay.

Single mutations to alanine for all residues had no significant effect, except for the R68A mutation, which increases the rhodopsin assay. Assays were generally in good agreement, although some differences of the GRKs. It is noteworthy that our findings with both limited to GRK5 and could be observed with other subfamilies 3, 9, and 10) propagates an effect on the kinase domain, rather than by a direct interaction with GPCRs. Consistent with this interpretation, helices 3 and 9 in GRK structures lie buried in part under the C-terminal extension (helix 11) such that if this were a part of the allosteric interaction domain, this helix must be displaced to accommodate GPCRs. In summary, this study provides new evidence for an important role of evolutionarily conserved sites in the kinase domain that are not required for GRK5 and -6 activity.

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