Palmitoylation Increases the Kinase Activity of the G Protein-Coupled Receptor Kinase, GRK6[†]

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ABSTRACT: The G protein-coupled receptor kinase GRK6 undergoes posttranslational modification by palmitoylation. Palmitoylated GRK6 is associated with the membrane, while nonpalmitoylated GRK6 remains cytosolic. We have separated palmitoylated from nonpalmitoylated GRK6 to assess their relative kinase activity. Palmitoylated GRK6 is 10-fold more active at phosphorylating β_2-adrenergic receptor than nonpalmitoylated wild-type GRK6 or a nonpalmitoylatable mutant GRK6. A nonpalmitoylatable mutant GRK6 which has been further mutated to undergo posttranslational geranylgeranylation is also more active, recovering most of the activity of the palmitoylated enzyme. This activity increase by lipid modification is expected, as the lipid helps GRK6 localize to cellular membranes where its receptor substrates are found. However, when assayed using a soluble protein (casein) as a substrate, both palmitoylated and prenylated GRK6 display significantly higher activity than nonpalmitoylated wild-type or nonpalmitoylatable mutant GRK6 kinases. This increased activity is not altered by addition of exogenous palmitate or phosphatidycholine vesicles, arguing that it is not due to direct activation of GRK6 by binding palmitate, nor to nonspecific association of the GRK6 with casein. Further, chemical depalmitoylation reduces the casein phosphorylation activity of the palmitoylated, but not prenylated, GRK6 kinase. Thus, palmitoylation of GRK6 appears to play a dual role in increasing the activity of GRK6: it increases the hydrophobicity and membrane association of the GRK6 protein, which helps bring the GRK6 to its membrane-bound substrates, and it increases the kinase catalytic activity of GRK6.

G protein-coupled receptor kinases (GRKs)[1] phosphorylate G protein-coupled seven transmembrane span receptors, which in turn allows the binding of an arrestin protein to the phosphorylated receptor, dampening receptor activation of heterotrimeric G proteins (*1*, *2*). Six distinct GRK enzymes are known currently, called GRK1 through GRK6, which can be divided into three distinct subfamilies based on sequence similarity, functional regulation, and gene organization (*2*−*4*). The central protein kinase catalytic domain of the GRKs is flanked by an amino-terminal domain which has been implicated as a regulatory and receptor substrate recognition domain (*5*−*9*) and a carboxyl-terminal domain which participates in localizing the kinases to the membrane (*10*−*12*). This membrane localization is essential for the activity of the GRKs, since their receptor substrates are located on the plasma membrane surface.

GRK1 (rhodopsin kinase) forms one subfamily of the GRKs, and contains a CAAX box motif at its carboxyl terminus which directs the posttranslational farnesylation and carboxymethylation of the protein (*13*). Following photoactivation of rhodopsin, GRK1 translocates from the rod outer segment cytosol to the disk membranes, where it phosphorylates activated rhodopsin (*14*). The farnesyl moiety, acting as a lipid anchor, helps localize the kinase to the membrane surface or perhaps to rhodopsin itself, allowing the kinase to more effectively phosphorylate light-bleached rhodopsin (*14*).

GRK2 and GRK3 (or βARK 1 and 2) form a second subfamily of GRKs. These kinases also translocate to the membrane surface in a receptor activation-dependent manner (*10*, *15*). Their mechanism differs from that of GRK1, however, in that both GRK2 and GRK3 contain pleckstrin homology (PH) domains in the carboxyl-terminal region which bind to G protein $\beta\gamma$ subunits (*10*, *12*), which are themselves anchored to the plasma membrane surface via a geranylgeranyl isoprenoid moiety (*16*). Agonist binding leads to receptor activation of G proteins and dissociation of the Gα subunit from the G$\alpha\beta\gamma$ heterotrimer. The free G$\beta\gamma$ subunits, together with membrane PIP_2 lipids, bind to the PH domain in the carboxyl terminus of GRK2 or -3, causing the translocation of these kinases to the membrane surface and allowing receptor phosphorylation (*10*, *11*).

GRK4, -5, and -6 form the third subfamily of GRKs. These differ from both the GRK1 and the GRK2 and -3

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[1] Abbreviations: β_2AR, β_2-adrenergic receptor; C-20, the 20 carbon (geranylgeranyl) isoprenoid; G protein, guanine nucleotide-binding regulatory protein; GRK, G protein-coupled receptor kinase; PH domain, pleckstrin homology domain.

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subfamilies in that they contain neither a CAAX box directing prenylation nor a G protein $\beta\gamma$ subunit-binding domain. The carboxyl terminus of GRK5 has a basic region which has been implicated in the membrane targeting of the kinase (*17*). Noncovalent binding of lipids to this region appears to increase the autophosphorylation of GRK5, possibly indicating activation of the kinase catalytic activity (*18*). We have shown that a cluster of cysteine residues in the carboxyl-terminal domain of GRK6 and of GRK4 is modified by palmitic acid (*19*, *20*). This lipid modification appears essential for the membrane association of the kinase, since palmitoylated GRK6 was found only in the membrane fraction, while nonpalmitoylated kinase remained soluble (*19*). More recently, it has been appreciated that GRK6 undergoes alternative splicing to generate three variants which differ in the sequence of their extreme carboxyl termini (*4*, *21*). Thus, while the originally identified GRK6A form undergoes palmitoylation (*19*), the GRK6B variant lacks the cysteine residues which are the sites of palmitoylation and in their place contains a sequence similar to the basic lipid-binding region of GRK5, and the GRK6C variant lacks both the sites of palmitoylation and the basic lipid-binding region (*4*).

The membrane localization and activity of the GRKs are dependent on their covalent modification by lipids (*14*) or noncovalent functional interactions with lipids (*6*, *11*, *18*). The GRKs rely on these lipid interactions and modifications for their ability to translocate to the membrane surface and for regulation of their activity (*6*, *11*, *12*). We have now investigated the role of palmitoylation in the regulation of GRK6A activity.

MATERIALS AND METHODS

Plasmids and Transfection of Mammalian Cells. pCMV5-GRK6, pCMV5-GRK6 $Cys^{561,562,565}Ser$ (GRK6 C→S triple mutant), and pCMV5-GRK6 $Cys^{561,562,565}Ser$-CVLL (GRK6 C-20 mutant) were prepared using standard procedures (22) from pCMV5-human GRK6A (*19*, *23*). Nearly confluent COS-7 cells on 150 cm^2 plates were transfected using lipofectamine (Gibco BRL), and cells were cultured as described previously (*19*). COS-7 cells were labeled with [^{3}H]palmitic acid or [^{35}S]cysteine and methionine mix (DuPont NEN) as previously described (*19*).

Preparation of Cytosolic and Membrane-Bound GRK6 from COS-7 Cells. COS-7 cells were harvested 48 h post-transfection in 20 mM Tris (pH 7.5) and 2 mM EDTA with protease inhibitors (10 μg/mL leupeptin, 20 μg/mL aprotinin, and 3 mM phenylmethylsulfonyl fluoride) and homogenized in a Dounce-type homogenizer (Wheaton). Unbroken cells were pelleted at 300*g* for 2 min. The remaining supernatant was centrifuged at 350,000*g* for 15 min to pellet the membranes. After centrifugation, the supernatant (cytosolic fraction) was removed, and the pellet (membrane fraction) was resuspended in 10 mM Tris (pH 7.5), 100 mM NaCl, and 2 mM EDTA (buffer A) containing 1.5% Triton X-100 to solubilize the membrane-bound GRKs. The resuspended pellet was mixed by rotation for 1 h at 4 °C and then centrifuged at 350000*g* for 15 min. The supernatant was applied to a 1 cm^3 Extracti-Gel D column (Pierce) to remove the detergent, and the flowthrough was concentrated using a Centricon-30 unit (Amicon). Samples were stored at 4 °C, and assays were performed within 1 week.

Western Blot Analysis. Samples were electrophoresed on 10% acrylamide gels in sodium dodecyl sulfate sample buffer and transferred to nitrocellulose membranes using the Novex XCell II Mini-Cell. The membranes were probed with a polyclonal antiserum produced in rabbits against a glutathione *S*-transferase fusion protein containing the last 114 amino acids of human GRK6A (*23*) as previously described for GRK5 (*17*). After being washed, the membranes were probed with an [^{125}I]-anti-rabbit IgG whole antibody from goat (DuPont NEN). The bands corresponding to the GRKs were quantified using a PhosphorImager (Molecular Dynamics) to estimate relative kinase concentrations.

Phosphorylation of β_2-Adrenergic Receptor and Casein. The human β_2AR was expressed in Sf9 cells, purified, and reconstituted into phospholipid vesicles containing 95% phosphatidylserine (Sigma) and 5% phosphatidylinositol 4,5-bisphosphate (Sigma) (*11*). Reconstituted receptor (40 nM) was incubated at 30 °C for 15 min with 7 μg of kinase extract, 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 2 mM EDTA containing 100 μM isoproterenol (Sigma) and 80 μM ATP in a total volume of 25 μL. Casein (30 μg) (Sigma) was incubated at 30 °C for 15 min with 7 μg of kinase extract, 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 2 mM EDTA containing 80 μM ATP in a total volume of 25 μL. Reactions were stopped with 25 μL of SDS sample-loading buffer containing dithiothreitol. The samples were electrophoresed on 10% acrylamide gels and dried. The amount of phosphorylation was quantified using a PhosphorImager (Molecular Dynamics). Phosphorylation data were then adjusted to account for differences in the kinase concentrations in the reaction cocktails, by comparing the amount of kinase in each extract relative to the amount of kinase in the wild-type membrane extract, based on quantitiative [^{125}I] Western blot analysis of the kinase extracts. Assays were linear in time through 20 min.

Hydroxylamine Treatment. Membrane extracts containing GRK6 wt or GRK6 C-20 mutant were incubated with 1 M hydroxylamine (pH 7.0) in buffer A or with 1 M Tris-HCl (pH 7.0) in buffer A for 60 min at 37 °C. The samples were concentrated with Centricon 30 centrifugal concentrators (Amicon), diluted with 1 mL of buffer A, and reconcentrated. This was repeated 3 times to remove the hydroxylamine. The protein concentrations were then determined and the samples used in phosphorylation assays.

RESULTS

Lipid-Modified GRK6 Has Enhanced Activity for Phosphorylating the β_2AR. Palmitoylation is the reversible posttranslational acylation of a protein with a 16-carbon-saturated fatty acid via a thioester or an oxyester bond. This modification, therefore, allows for the potential regulation of proteins through cycles of palmitoylation and depalmitoylation. In this regard, palmitate turnover has been shown to occur after receptor activation of the β_2-adrenergic receptor (*24*), the α_2A-adrenergic receptor (*25*), nitric oxide synthase (*26*), and the α subunits of heterotrimeric G proteins (*27*).

To determine whether this lipid modification plays a role in regulating the activity of GRK6A, we examined the ability of palmitoylated GRK6 to phosphorylate the β_2-adrenergic receptor (β_2AR) relative to the nonpalmitoylated form of the

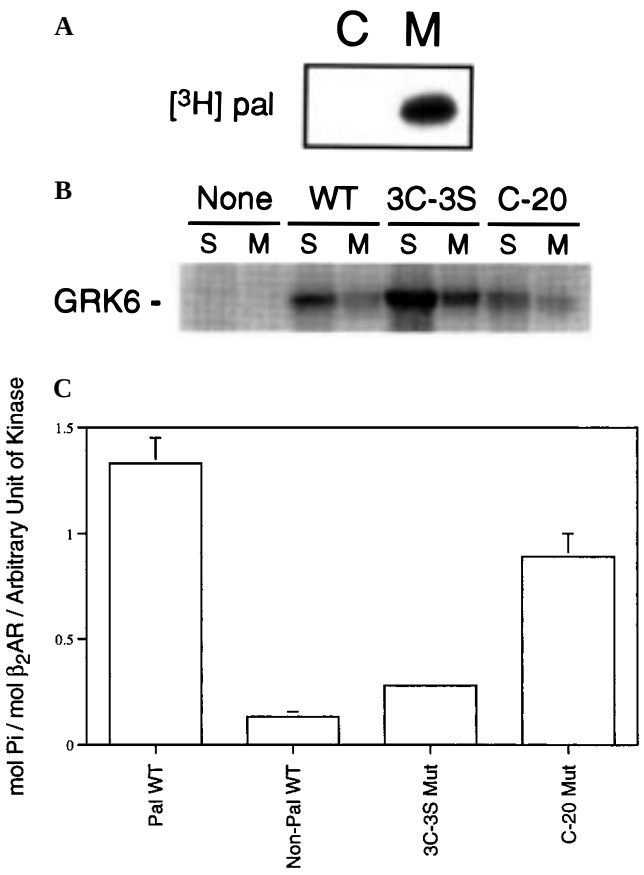

FIGURE 1: Phosphorylation of the β_2AR by lipid-modified forms of GRK6. (A) COS-7 cells were transfected with the cDNA encoding wild-type GRK6A and labeled with [^{3}H]palmitic acid. Membrane-bound (M) and cytosolic (C) GRK6 were separated as described under Materials and Methods prior to immunoprecipitation. Exposure time was 3 months. (B) Equal volumes of COS-7 cell membrane extract (M) and cytosolic samples (S) from cells overexpressing wild-type or mutant forms of GRK6 were separated by SDS−PAGE and immunoblotted using anti-GRK6 antiserum and ^{125}I-labeled secondary antibody. Following overnight exposure to a PhosphorImager plate, the intensity of the individual bands was quantified. A representative PhosphorImager scan is shown, but a similar assay was performed for every extract preparation. WT, wild-type GRK6; 3C-3S, C→S triple mutant GRK6; C-20, geranylgeranylated C→S triple mutant GRK6. (C) COS-7 cell membrane and cytosolic extracts containing GRK6 were used to phosphorylate 1 pmol of β_2AR for 15 min. The data were adjusted to equalize the amount of GRK6 present in each extract, relative to wild-type palmitoylated kinase, based on Western blot analysis like that shown in panel B. Pal, wild-type palmitoylated GRK6; Non-Pal, wild-type nonpalmitoylated GRK6; 3C-3S Mut, C→S triple mutant GRK6; C-20 Mut, geranylgeranylated C→S triple mutant GRK6. Results shown represent the mean values obtained from at least three separate determinations.

kinase. We had previously shown that in Sf9 cells the nonpalmitoylated GRK6 is found in the cytosol, while palmitoylated GRK6 is found exclusively in the membrane fraction (*19*). Palmitoylated GRK6 was found only in the membrane fraction when COS-7 cells were labeled with [^{3}H]-palmitic acid (Figure 1A).

To isolate the two forms of the kinase, COS-7 cells were transfected with the wild-type GRK6 cDNA and homogenized, and the cytosolic and membrane fractions were separated by centrifugation. The membranes were solubilized with 1.5% Triton X-100 to extract the palmitoylated GRK6, the detergent was removed with an Extracti-gel D column, and the membrane extract was concentrated. The amount of GRK6 in the membrane extract and soluble fractions was quantified by immunoblotting with a GRK6 antiserum and ^{125}I-labeled secondary antibody, which was detected following exposure to PhosphorImager plates (Figure 1B). Cytosolic and membrane extracts were tested for their ability to phosphorylate the β_2AR (Figure 1C). Palmitoylated GRK6 was found to be 10-fold more active in phosphorylating the β_2AR than nonpalmitoylated GRK6.

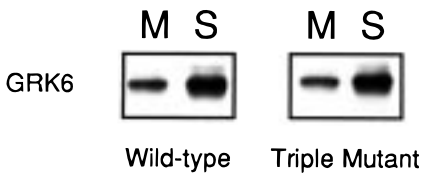

FIGURE 2: Membrane/cytosol fractionation of palmitoylated and nonpalmitoylatable mutant forms of GRK6. COS-7 cells were transfected with the cDNA encoding wild-type GRK6 or triple mutant GRK6, and incubated with ^{35}S-labeled amino acids to label proteins. Membrane-bound (M) and soluble (S) GRK6 were separated as described under Materials and Methods prior to immunoprecipitation. Exposure time was for 24 h.

The increased kinase activity of palmitoylated GRK6 is presumably due to the palmitoyl moiety anchoring the kinase to the membrane. This increased activity, however, may have been caused by other factors, such as activation of palmitoylated GRK6 through its association with the membrane or through interactions with an activating protein present in the membrane extract but not in the cytosolic extract. To distinguish activation through association of the kinase with the membrane from activation through potential protein−protein interactions found only in the membrane extract, the activity of the GRK6 Cys^{561,562,565}Ser mutant (triple mutant), which is not palmitoylated (19), was tested. The triple mutant GRK6 was isolated from the membrane fraction using Triton X-100. This was possible since a fraction of the triple mutant kinase was associated with the membrane when overexpressed in COS-7 cells, even though it was not acylated (Figure 2)(*19*). This is due to the fact that the mutant kinase retains the sequence in its amino-terminal domain which allows it to bind noncovalently to PIP_2 lipids, in a manner analogous to GRK5 (*6*). The triple mutant showed comparable activity to the nonpalmitoylated wild-type kinase in phosphorylating the β_2AR (Figure 1C), indicating that the lipid modification was indeed responsible for the increased activity of the palmitoylated kinase, rather than other factors in the membrane extract.

We next explored whether the increased activity was due to palmitoylation specifically or if a distinct lipid modification also could increase the activity of the kinase by increasing anchoring to the membrane. We generated an isoprenylated version of the C→S triple mutant GRK6 containing the geranylgeranyl isoprenoid by engineering a CAAX box with the amino acid sequence CVLL on the carboxyl-terminal end of the GRK6 triple mutant sequence (C-20 mutant GRK6) (*14*). The isoprenylated C-20 mutant isolated from membranes showed 80% of the wild-type palmitoylated GRK6 activity (Figure 1C), which is significantly more than the activity of the nonpalmitoylated GRK6. This result suggests that the increased activity was not due to palmitoylation specifically, but results primarily from lipid-dependent anchoring of the kinase to the membrane surface, which brings the kinase into closer proximity to its receptor

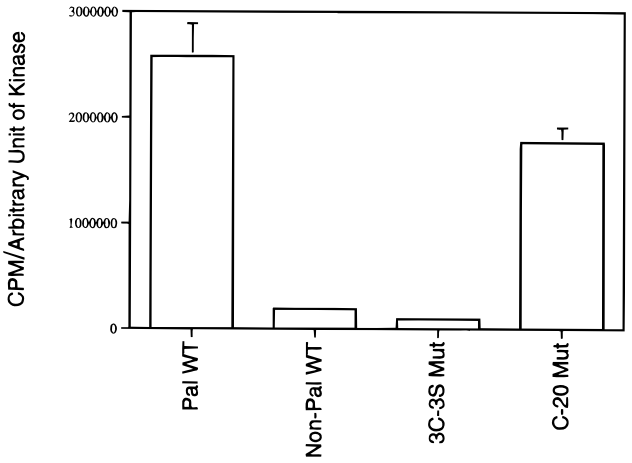

FIGURE 3: Phosphorylation of casein by lipid-modified forms of GRK6. COS-7 cell membrane and cytosolic extracts containing GRK6 were used to phosphorylate 25 μg of casein for 15 min. To determine the amount of kinase present in the cytosolic and membrane extracts, [^{125}I] Western blot analysis was performed using primary antisera raised against the C-terminus of GRK6. The data were adjusted to equalize the amount of kinase present in each extract relative to wild-type palmitoylated kinase. Pal, wild-type palmitoylated GRK6; Non-Pal, wild-type nonpalmitoylated GRK6; 3C-3S Mut, triple mutant GRK6; C-20 Mut, geranylgeranylated triple mutant GRK6. Results shown represent the mean values obtained from at least four separate determinations.

substrates. These data are consistent with those found for GRK1 and GRK2. Prenylation of GRK1 significantly increases the activity of the kinase for phosphorylating rhodopsin (*14*). Similarly, the protein−protein interaction of GRK2 with the geranylgeranylated $\beta\gamma$ subunits of heterotrimeric G proteins, in concert with direct binding to PIP_2 in the membrane, leads to an increase in activity toward the β_2AR (*10*, *11*).

Lipid-Modified GRK6 Has Enhanced Activity for Phosphorylating Casein. We next characterized the different lipid-modified GRK6 preparations for their ability to phosphorylate the soluble protein casein, which is known to be a substrate for GRK6 (*28*). Surprisingly, the results for the phosphorylation of casein were similar to the β_2AR results (Figure 3). That is, the palmitoylated GRK6 was 12-fold more active in phosphorylating casein than the nonpalmitoylated GRK6. In this assay, both casein and the GRK6 are soluble, which suggests that the increased activity of the palmitoylated kinase is due to activation by the covalent lipid modification.

To distinguish the activity effect of palmitoylation from possible activation of the palmitoylated kinase through other factors present in the membrane fraction but not in the cytosolic fraction, the triple mutant (isolated from the membrane fraction) also was used to phosphorylate casein. The triple mutant showed comparable activity to the wild-type nonpalmitoylated GRK6 (Figure 3), indicating that the increased activity was due to palmitoylation of the kinase. The prenylated C-20 mutant GRK6 was also tested to determine if prenylation could increase the activity of the kinase in phosphorylating casein. The prenylated C-20 mutant GRK6 showed 70% of the palmitoylated wild-type activity, and significantly more than the nonpalmitoylated wild-type activity (Figure 3). These data, taken together with the β_2AR phosphorylation data, indicate that palmitoylation

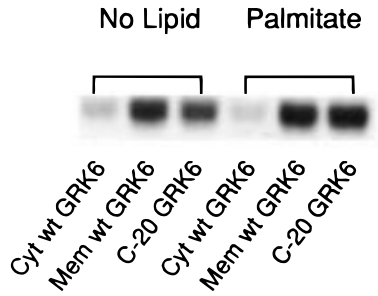

FIGURE 4: Casein phosphorylation by lipid-modified forms of GRK6 in the presence of lipid vesicles. Extracts containing wild-type nonpalmitoylated GRK6 (Cyt wt), wild-type palmitoylated GRK6 (Mem wt), or geranylgeranylated triple mutant GRK6 (C-20) were used to phosphorylate 25 μg of casein in the presence of palmitic acid vesicles (Pal) or in the absence of added lipid (No Lipid). Results shown are representative of three experiments.

and artificial prenylation not only increase the activity of the kinase by anchoring it to the membrane but also potentially activate the kinase.

In the β_2AR assay, the palmitoylated GRK6 associates with the membrane surface due to its lipid modification, and at least to some extent is more active since it is in closer proximity to the receptor substrate. In the soluble casein assay, however, there can be no membrane association effect for the kinase, and the increased activity of lipid-modified GRK6 is due to activation of the kinase by its lipid modification. It is possible that the catalytic activation of the kinase evident in the casein assay is also present in the β_2-AR assay. However, it would be difficult to separate potential catalytic activation of the kinase by its lipid modification from the increased activity obtained through anchoring the kinase to the membrane in the β_2AR assay.

Covalent Lipid Modification of GRK6 Activates the Kinase. We next explored whether the actual covalent modification of the kinase leads to the activity increase or if free lipid alone could activate the kinase. To determine if free palmitic acid could activate GRK6, the wild-type nonpalmitoylated kinase was assayed for its ability to phosphorylate casein in the presence of exogenous palmitic acid (Figure 4). Addition of palmitic acid had no effect on phosphorylation by wild-type nonpalmitoylated GRK6, indicating that the lipid must be covalently attached to increase the activity of GRK6. Further, the phosphorylation activity of palmitoylated or geranylygeranylated mutant GRK6 was not altered by addition of exogenous palmitate.

In the soluble casein phosphorylation assay (Figure 3), the palmitoyl moiety covalently linked to the kinase could be artificially increasing the activity of the kinase because of the aqueous environment. That is, there is no lipid environment in the assay to stabilize the GRK6-linked palmitic acid, and the hydrophobicity of the palmitic acid could force the lipid to sequester onto the surface of the casein protein, causing the appearance of a more active kinase. We thus added phosphatidylcholine vesicles to the casein assay to simulate a membranous environment and to provide a hydrophobic area for the lipid to sequester. The addition of the vesicles had no effect on the ability of wild-type palmitoylated GRK6 or prenylated C-20 mutant GRK6 to phosphorylate casein (data not shown). These data, taken

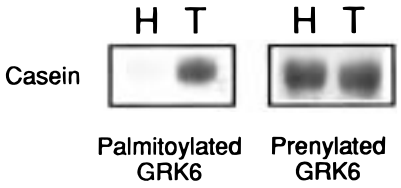

FIGURE 5: Phosphorylation of casein after hydroxylamine treatment of lipid-modified forms of GRK6. Extracts containing wild-type palmitoylated GRK6 (Palmitoylated) or geranylgeranylated triple mutant GRK6 (Prenylated) were used to phosphorylate 25 μg of casein. Before phosphorylation assays, kinase extracts were treated with either 1 M hydroxylamine (H) or 1 M Tris-HCl (T) as described under Materials and Methods. Results shown are representative of three experiments.

all together, suggest that the palmitic acid activates the kinase directly through its covalent modification of GRK6.

Hydroxylamine Treatment Dampens the Activity of Palmitoylated GRK6. To more firmly establish that palmitoylation activates GRK6, the palmitoyl moiety was chemically removed from the protein, and the kinase activity was tested in the soluble casein assay. Palmitic acid linked through a thioester bond can be hydrolyzed in neutral hydroxylamine (*29*), and we have previously demonstrated chemical depalmitoylation of GRK6 (*19*). To assess the functional consequences of removal of the palmitoyl moiety, the wild-type membrane kinase extract was incubated with 1 M hydroxylamine (pH 7) or in 1 M Tris buffer (pH 7) as a control. The two treated samples were then used in casein phosphorylation assays. Hydroxylamine depalmitoylation significantly dampened the activity of wt GRK6 as compared to the palmitoylated GRK6 control sample treated with Tris buffer (Figure 5). Prenylated C-20 mutant GRK6 samples also were treated with 1 M hydroxylamine or with 1 M Tris buffer. Hydroxylamine treatment cannot remove the stably linked prenyl lipid, and had no effect on the ability of the prenylated C-20 mutant GRK6 to phosphorylate casein (Figure 5). This indicates that hydroxylamine treatment per se is not detrimental to GRK6 catalytic activity. These data strongly suggest that palmitoylation activates GRK6 directly, since chemical removal of the palmitic acid significantly reduces the activity of the kinase to phosphorylate the soluble substrate casein.

DISCUSSION

In the GRK family, several mechanisms for the membrane association of the kinases have evolved. Each mechanism is dependent on the interaction with lipids, either covalent or noncovalent. These lipid interactions also allow for the regulation of GRK activity. GRK1 is prenylated with the farnesyl isoprenoid, and this modification is essential for its ability to translocate to the membrane in response to light activation of rhodopsin, which results in its enhanced activity (*14*). Likewise, GRK2 is dependent upon protein−protein interactions with prenylated G protein $\beta\gamma$ subunits, in concert with direct interaction with membrane PIP_2, for membrane association and for enhanced activity (*10*, *11*). GRK5 associates with the membrane by binding lipids noncovalently in a manner that increases the catalytic activity and autophosphorylation of the kinase (*6*, *18*).

We now show that wild-type palmitoylated GRK6 is 10 times more active than wild-type nonpalmitoylated GRK6 for phosphorylating the β_2AR. Palmitoylated GRK6 is also 12 times more active than nonpalmitoylated GRK6 in phosphorylating casein. A nonpalmitoylatable mutant GRK6 has lower activity, similar to the wild-type nonpalmitoylated GRK6. However, increased activity can be restored by replacement of palmitate with nearby C-20 prenylation. These data indicate that palmitoylation increases GRK6 activity not only by anchoring it to the membrane and bringing it in closer proximity to its receptor substrate, but also by activating the kinase catalytic activity. When compared to other purified GRKs, GRK6 has appeared to be substantially less active in phosphorylating model receptors, such as rhodopsin and the β_2AR (*28*, *30*). The present results indicate that the activated form of GRK6, the small membrane-targeted, palmitoylated pool of enzyme, is significantly more active than the cytosolic, nonpalmitoylated form. Thus, GRK6, if assayed as the receptor might actually see it, is probably not so different in absolute activity from other GRKs.

It is puzzling why both palmitoylation and artificial prenylation activate the kinase. The site of prenylation is 11 amino acids downstream from the site of palmitoylation in the carboxyl terminus of the kinase. It is possible that the proximity of the two modifications on the polypeptide chain may cause them to act in a similar manner, resulting in kinase activation. The degree of activation, however, is higher for palmitoylation than it is for isoprenylation. This may be a result of the distance between the two modifications, of intrinsic differences between the two lipids, or of the potential for multiple palmitate modifications of a single GRK6 molecule compared to a single prenyl chain modification.

In a similar study, Loudon and Benovic (*31*) recently reported that palmitoylation or artificial prenylation increases the activity of GRK6 to phosphorylate the β_2AR. However, they reported that these lipid-modified GRK6 proteins do not have an increased ability to phosphorylate a soluble substrate. There are significant methodological differences between their study and the one we report here which contribute to our differing conclusions. Whereas we have separated the palmitoylated and nonpalmitoylated wild-type GRK6 proteins to allow their direct comparison, Loudon and Benovic (*31*) used the two forms together in a mixture by extracting the cells directly with detergent. Since there is much more soluble, nonpalmitoylated GRK6 than membrane-bound, palmitoylated GRK6 in our cells (see Figure 2), the activity of the mixture significantly underrepresents the higher activity of the palmitoylated GRK6. We have explicitly compared the membrane-derived samples of all lipid-modified variants of GRK6, and under our conditions, lipid-modified forms of GRK6 are more active against both receptor and soluble substrates. On the other hand, Loudon and Benovic (*31*) immunoprecipitated the total cellular pools of the wild-type and a nonpalmitoylatable mutant GRK6 using a carboxyl-terminal anti-peptide antibody, and find no difference in kinase activity toward the soluble substrate phosvitin. However, they state that the C-20 prenyl-modified mutant kinase does not immunoprecipitate under their conditions. Since the palmitate and prenyl lipids are located quite close together in the GRK6 carboxyl terminal and are both near the peptide antigen site, it is of some concern that they did not demonstrate that the palmitoylated form of the

wild-type GRK6 can be immunoprecipitated at all. This leaves the distinct possibility that they preferentially immunoprecipitated only the nonpalmitoylated pool of wild-type GRK6, and are thus comparing the nonpalmitoylated form of the wild-type GRK6 to the nonpalmitoylatable mutant GRK6. Thus, while we agree with the conclusions of Loudon and Benovic (*31*) that palmitoylation of GRK6 increases its membrane association and receptor phosphorylation activity, we also believe that palmitoylation increases the catalytic activity of the kinase, as evidenced by increased phosphorylation of the soluble substrate casein (Figure 3) which can be reduced by chemical removal of the palmitate (Figure 5).

Recently, palmitoylation has been linked to conformational changes in the gramicidin transmembrane channel (*32*). Acylation, in this case, leads to changes in the orientation of side groups around the site of palmitoylation and results in a longer lifetime of the gramicidin dimer. Palmitoylation could be acting in a similar manner for GRK6 by altering the orientation of side chains around the site of palmitoylation and activating the kinase, or, alternatively, by interacting with some distant hydrophobic surface on the kinase.

Lipid modifications are an essential part of several signal transduction pathways (*16*). Palmitoylation is important for the activity of a variety of signaling proteins, by playing a role in regulating membrane association and subcellular localization. Thus, for endothilial nitric oxide synthase (*26*, *33*, *34*), several src family nonreceptor tyrosine kinases (*35*−*37*), and several G protein α-subunits (*27*, *38*, *39*), palmitoylation directs membrane association, caveolar localization, and increased functional activity. Receptor activation leads to depalmitoylation, membrane dissociation, and inactivation for eNOS (*26*), and $G_s\alpha$ (*27*, *39*). For GAP 43, depalmitoylation increases its activation of G_o (*40*). In this case, palmitoylation may regulate a cycle of palmitoylated membrane-bound inactive GAP 43 and nonpalmitoylated cytosolic active GAP 43.

Palmitoylation allows for the potential dynamic regulation of GRK6A. We envisage a cycle in which inactive GRK6A translocates to the plasma membrane in a signal-dependent manner, where it would be palmitoylated by palmitoyltransferase, becoming anchored and activated. Alternatively, GRK6A may be palmitoylated in the endoplasmic reticulum and shuttled to the plasma membrane, where it phosphorylates its receptor substrate in an agonist-dependent manner. Whether palmitoylated GRK6A has a preference for localizing to caveolae is unknown. Depalmitoylation would then result in a return to an inactive cytosolic kinase. Nonpalmitoylatable variants of GRK6, such as GRK6B and GRK6C, would lack such a palmitoylation−depalmitoylation regulatory cycle. The means for the regulation of the membrane association and activity of these other two GRK6 forms remain to be explored.

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