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Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes

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G protein-coupled receptor (GPCR) kinases (GRKs) are critical regulators of cellular signaling and function. In cardiomyocytes, GRK2 and GRK5 are two GRKs important for myocardial regulation, and both have been shown to be up-regulated in the dysfunctional heart. We report that increased levels and activity of GRK5 in failing myocardium may have unique significance due to its nuclear localization, a property not shared by GRK2. We find that transgenic mice with elevated cardiac GRK5 levels have exaggerated hypertrophy and early heart failure compared with control mice after pressure overload. This pathology is not present in cardiac GRK2-overexpressing mice or in mice with overexpression of a mutant GRK5 that is excluded from the nucleus. Nuclear accumulation of GRK5 is enhanced in myocytes after aortic banding *in vivo* and *in vitro* in myocytes after increased Gαq activity, the trigger for pressure-overload hypertrophy. GRK5 enhances activation of MEF2 in concert with Gq signals, demonstrating that nuclear localized GRK5 regulates gene transcription via a pathway critically linked to myocardial hypertrophy. Mechanistically, we show that this is due to GRK5 acting, in a non-GPCR manner, as a class II histone deacetylase (HDAC) kinase because it can associate with and phosphorylate the myocyte enhancer factor-2 repressor, HDAC5. Moreover, significant HDAC activity can be found with GRK5 in the heart. Our data show that GRK5 is a nuclear HDAC kinase that plays a key role in maladaptive cardiac hypertrophy apparently independent of any action directly on GPCRs.

Desensitization of seven-transmembrane–spanning G protein-coupled receptors (GPCRs) is triggered by agonist-dependent phosphorylation via a family of serine/threonine protein kinases known as GPCR kinases (GRKs). Seven GRK family members have been identified, and outside of GRK1 (rhodopsin kinase) and GRK7, which are localized to the retina, GRKs are ubiquitously expressed. GRK2 and GRK5 are abundantly expressed in myocardium, and they have been found to be key regulators of cardiac signaling and function (1). Adding to their importance in the heart is the fact that both are found to be up-regulated during pathological myocardial conditions, including in the failing human heart (2, 3). Increased GRK activity in the compromised heart would lead to enhanced desensitization of GPCRs, some of which may protect the heart from further damage and promote an adaptive phenotype; however, some overly desensitized GPCRs such as β-adrenergic receptors (β-ARs) also appear to become maladaptive, contributing to pathogenesis through the loss of inotropic reserve (3).

In the heart, GRK2 and GRK5 appear to have both overlapping (e.g., β-ARs) and distinct GPCR substrates (4, 5). These two GRKs appear also to have differences concerning induction of specific downstream signaling events after phosphorylating select GPCRs because GRK5 activity, but not GRK2 activity, toward α_1-adrenergic receptors (α_1-ARs) can lead to transactivation of the tyrosine kinase receptor for epidermal growth factor in myocytes (6). These differences between GRK2 and GRK5 are not surprising because they have specific structural, localization, and regulatory properties. GRK2, a member of the β-AR kinase subfamily of GRKs, is primarily cytosolic, and its membrane and GPCR recruitment are dependent on binding to G protein $\beta\gamma$-subunits (1, 7). GRK5, on the other hand, a member of the GRK4-like subfamily, is primarily membrane bound (1, 7). Moreover, it has recently been shown that members of the GRK4 subfamily have a functional nuclear localization sequence (NLS) (8), and, indeed, GRK5 can be found in the nucleus of cells, including cardiac myocytes (8, 9); however, its regulation and role within the nucleus are not understood. Finally, to highlight differences between GRK2 and GRK5, they have recently been found to interact with distinct non-receptor proteins in cells (10–14); however, any potential (patho)physiological roles of non-GPCR functions of GRKs have not been defined.

Compared with up-regulated GRK2, which has been shown to play a critical role in the pathogenesis of heart failure (HF) (1, 3, 7), the role of enhanced GRK5 in compromised myocardium has not been explored in depth. We have previously created transgenic mice with cardiomyocyte-specific GRK5 overexpression (4, 5). These transgenic GRK5 mice (TgGRK5), like cardiac GRK2 transgenic mice (TgGRK2) (15), have desensitized *in vivo* β-AR responses; however, these TgGRK5 mice have yet to be stressed to determine the cardiac consequences and significance of elevated GRK5. Therefore, in this study, we first used a model of transverse aortic constriction (TAC) to determine whether increased cardiac GRK5 levels can influence myocardial responses to ventricular pressure overload and subsequent hypertrophy. We found that compared with nontransgenic littermate control (NLC) mice and TgGRK2 mice that behaved as NLCs, TgGRK5 mice have an exaggerated hypertrophic response and a rapid decrease in cardiac function leading to early HF and death. Additionally, we found that mice with overexpression of a mutant nuclear-deficient GRK5 (Tg-ΔNLS) in cardiomyocytes no longer had the pathology observed in TgGRK5 mice. We found that in response to pressure overload and hypertrophic stimuli, GRK5 accumulates in the nucleus of myocytes, which correlates with enhanced ventricular expression of the hypertrophy-associated fetal gene program. Mechanistically, we demonstrate that this is due to nuclear GRK5 acting as a kinase for the class II histone deactylase-5 (HDAC5), a major transcriptional repressor and nodal regulator of cardiac hypertrophy (16, 17). Our data provide unique evidence that GRK5

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The authors declare no conflict of interest.

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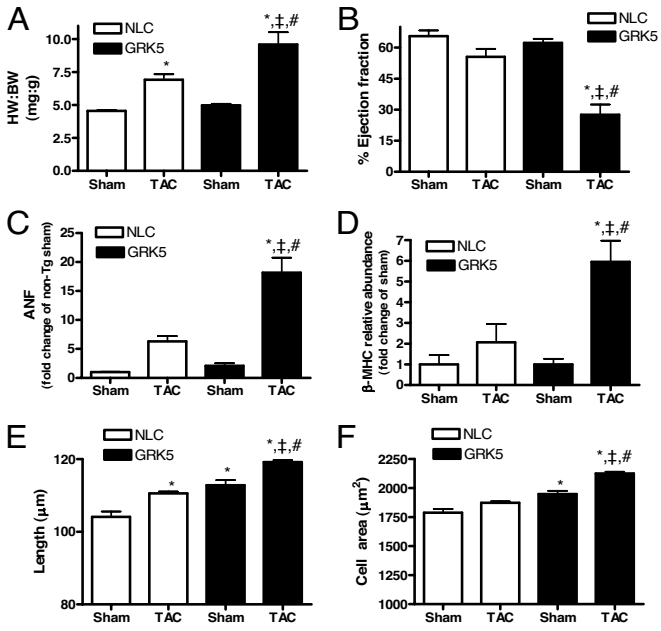

Fig. 1. GRK5 potentiates pressure-overload cardiac hypertrophy *in vivo*. (*A*) Cardiac hypertrophy as determined by HW/BW ratios 4 weeks post-TAC in NLC mice (sham and TAC, $n = 8$ each) and TgGRK5 mice (sham and TAC, $n = 9$ each). (*B*) Ejection fraction 4 weeks post-TAC in these mice. (*C* and *D*) Real-time quantitative RT-PCR data from mRNA from the left ventricles of NLC ($n = 4$) and TgGRK5 mice ($n = 5$) 2 weeks post-TAC. Atrial natriuretic factor (ANF) (*C*) and β-myosin heavy chain (β-MHC) (*D*) are normalized to 28S RNA. (*E* and *F*) Myocyte size is increased in TgGRK5 mice post-TAC compared with post-TAC NLC control mice. All values are calculated from the average of 50 cells from $n = 3$ animals. All data presented above are the mean ± SEM. *, $P < 0.05$ versus NLC sham; ‡, $P < 0.05$ versus GRK5 sham; #, $P < 0.05$ versus NLC post-TAC (one-way ANOVA, Bonferroni's multiple comparison test).

has distinct functions independent from its membrane localization and actions on GPCRs and that this nuclear HDAC kinase activity can have significant *in vivo* pathophysiological consequences.

Results

Cardiac GRK5 Overexpression Augments Hypertrophy *in Vivo*. We subjected TgGRK5 and NLC mice to TAC. Interestingly, we found that TgGRK5 mice had an exaggerated hypertrophic response compared with control mice, because there was significantly higher heart weight-to-body weight (HW/BW) ratios 4 weeks post-TAC (Fig. 1*A*). We also found that TgGRK5 mice developed significant left ventricle dysfunction after TAC compared with NLC mice (Fig. 1*B*) with higher mortality (data not shown). Several other *in vivo* physiological parameters also demonstrate early HF in TgGRK5 mice compared with NLC mice [see supporting information (SI) Table S1], which is published as supporting information on the PNAS web site]. This intolerance to TAC and enhanced hypertrophy appears to be GRK5 specific, because cardiac-GRK2 transgenic mice displayed morphological and functional responses identical to NLC mice after 4 weeks of TAC (data not shown).

TgGRK5 mice also showed an accelerated hypertrophic response after just 2 weeks of TAC, because this was shown by both cardiac mass measurements [HW/BW ratio (mg/g); NLC = 5.3 ± 0.13 ($n = 5$); TgGRK5 = 6.9 ± 0.61 ($n = 4$), $P < 0.05$] and significantly enhanced ventricular expression of atrial natriuretic factor (Fig. 1*C*) and β-myosin heavy chain (Fig. 1*D*), two key "fetal genes" associated with hypertrophied myocardium. Moreover, isolated adult cardiomyocytes from these mice showed a significant increase in cell length (Fig. 1*E*) and total area (Fig. 1*F*) in TgGRK5 myocytes compared with NLC cells.

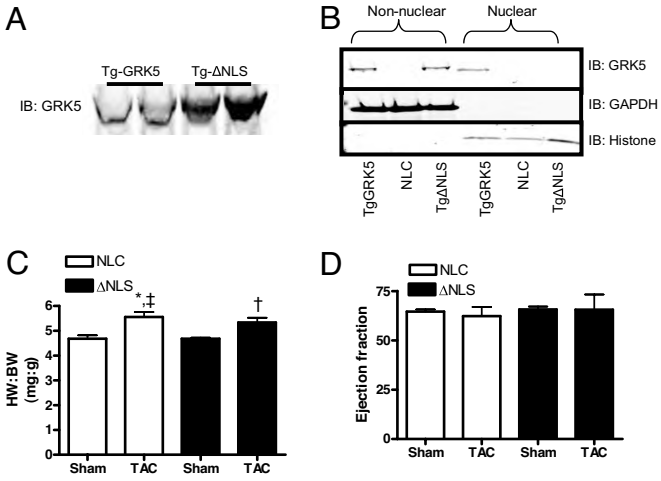

Fig. 2. Nuclear deficient GRK5 transgenic mice display normal pathology after TAC. (*A*) Western blot for GRK5 from whole heart lysates isolated from TgGRK5 and TgGRK5ΔNLS mice. (*B*) Nuclear and nonnuclear fractions were prepared from whole hearts isolated from TgGRK5, NLC, and TgGRK5ΔNLS mice, and GRK5 was visualized by Western blot analysis. Endogenous GRK5 in NLC hearts was observed on longer exposure (data not shown). (*C* and *D*) TgGRK5ΔNLS mice (sham, $n = 14$; TAC, $n = 5$) or NLC mice (sham, $n = 19$; TAC, $n = 7$) were subjected to pressure overload. Heart weight-to-body weight ratio (HW/BW) (*C*) and left ventricular function (ejection fraction) (*D*). *, $P < 0.05$ versus NLC sham; ‡, $P < 0.05$ versus ΔNLS sham (one-way ANOVA, Bonferroni's multiple comparison test); †, $P < 0.05$ versus sham (two-tailed *t* test).

Nuclear GRK5 Is Responsible for Pathology after TAC. Next, we created cardiac-targeted GRK5ΔNLS (nuclear deficient GRK5) transgenic mice (TgΔNLS) with levels of myocardial overexpression of this GRK5 mutant comparable with WT GRK5 overexpression in TgGRK5 mice (TgΔNLS, 22-fold over endogenous GRK5; TgGRK5, 21-fold over endogenous GRK5) (Fig. 2*A* and *B*). This mutant GRK5 has its NLS mutated and was shown to be excluded from the nucleus of cells (8), a result we also found in these transgenic hearts (Fig. 2*B*). After 4 weeks of TAC, we observed an increase in HW/BW ratios in both the control and TgΔNLS hearts; however, unlike in TgGRK5 mice, there was no significant change between groups (Fig. 2*C*). In addition, there was no significant change in ejection fraction 4 weeks after TAC between control mice and TgΔNLS (Fig. 2*D* and Table S2), which is quite different from when WT GRK5 is overexpressed in the heart (Fig. 1*B*). These results indicate a critical role of nuclear GRK5 localization in maladaptive hypertrophy and the progression of HF.

Pressure Overload Causes GRK5 Nuclear Accumulation *in Vivo*. We next began to explore potential mechanisms responsible for this GRK5-specific *in vivo* pathological phenotype. Our exploration began in the nucleus of cardiomyocytes, where processes that included increased gene expression play a key role in supporting the growth of the heart (17, 18). Therefore, we first explored the distribution of GRK5 in hypertrophied mouse hearts. This is potentially relevant, because up-regulated GRK5 in compromised myocardium as in human HF would also be expected to lead to increased nuclear GRK5 localization. Indeed, compared with NLC myocytes, TgGRK5 myocytes have more GRK5 present in the nucleus (Fig. 3*A*). To determine whether the localization pattern of GRK5 changes in response to pressure overload, we isolated cardiomyocytes from sham and 2-week post-TAC TgGRK5 mice and prepared nuclear and nonnuclear fractions. We found that TAC induced significant (≈2-fold) nuclear GRK5 accumulation (Fig. 3 *B* and *C*), suggesting that nuclear GRK5 activity may play a maladaptive role in the responses to pressure overload.

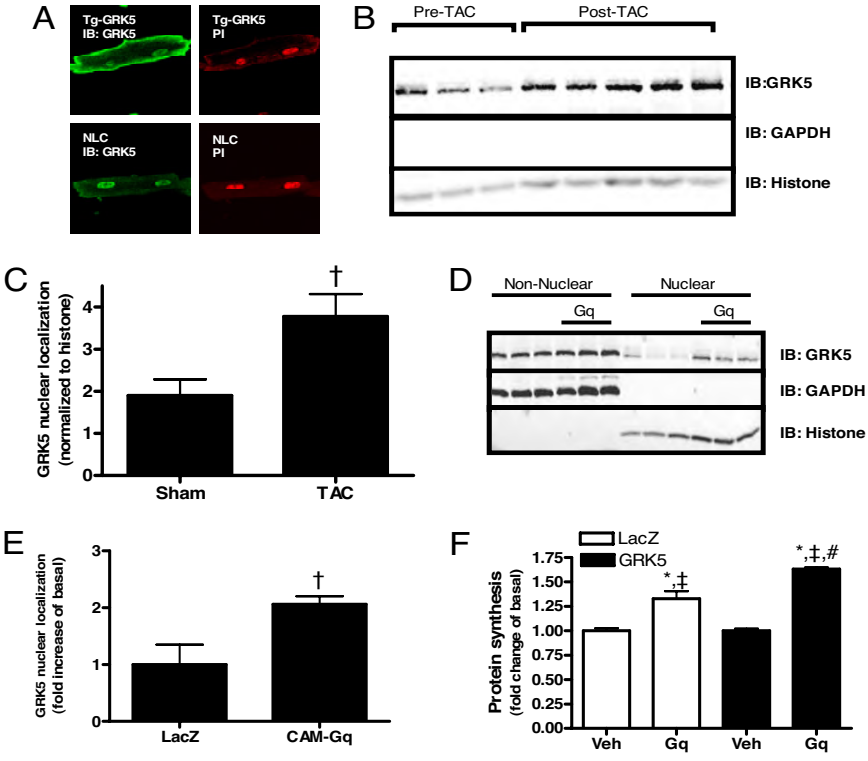

Fig. 3. GRK5 accumulates in the nucleus of myocytes after hypertrophic stimuli and enhances Gq-mediated cellular growth. (*A*) Adult cardiomyocytes were isolated, and immunofluorescent images are shown on representative myocytes ($n = 4$ preparations each) from TgGRK5 and NLC hearts. The GRK5 is shown in green and is observed at the plasma membrane and in the nucleus. Propidium iodide was used as a nuclear control (shown in red). (*B* and *C*) Nuclear and nonnuclear fractions (data not shown) were prepared from adult myocytes, and GRK5 localization was visualized by Western blot analysis, by using GAPDH and histone as loading controls for nonnuclear and nuclear fractions, respectively. (*D* and *E*) Western blot for GRK5, GAPDH, and histone of nonnuclear and nuclear fractions from NRVMs infected with Ad-GRK5 along with Ad-LacZ or Ad-CAM-Gαq. (*F*) [^{3}H]Phenylalanine incorporation was measured in NRVMs from either Ad-LacZ- or Ad-GRK5-treated cells. All data presented above are the mean ± SEM. *, $P < 0.05$ versus LacZ veh; ‡, $P < 0.05$ versus GRK5 veh; #, $P < 0.05$ versus NLC plus CAM-Gq (one-way ANOVA, Bonferroni's multiple comparison test); †, $P < 0.05$ versus sham (two-tailed *t* test) ($n = 3$ separate experiments).

Gq Activation Causes Nuclear GRK5 Accumulation and Hypertrophy *in Vitro*. The potential mechanistic role of this nuclear accumulation of GRK5 after TAC was next explored *in vitro* in myocytes. It is known that pressure overload induces ventricular hypertrophy via signaling pathways downstream of the heterotrimeric G protein, Gq (19). In fact, after GPCR activation, several signals converging at the level of activation of Gq can induce cardiac hypertrophy (18). This includes nuclear signals downstream of Gq activation leading to enhanced gene expression dependent on the activation of MEF2 (20, 21). Moreover, sustained signaling through Gq has been shown to be the predominant pathway leading to maladaptive hypertrophy and subsequent HF (18). Thus, we examined whether nuclear GRK5 could serve as a link between Gq activation and hypertrophic gene expression and potentially be responsible for the exaggerated post-TAC phenotype observed in TgGRK5 mice. We first infected neonatal rat ventricular myocytes (NRVMs) with an adenovirus containing WT GRK5 (AdGRK5) with coinfection of a constitutively active mutant (CAM) of Gαq (22) of or an adenovirus containing β-galactosidase (AdLacZ) as a control. We found that after myocyte Gq activation, GRK5 nuclear accumulation was significantly increased ≈2-fold (Fig. 3 *D* and *E*). NRVMs treated with phenylephrine, an agonist for α_1-ARs, also showed increased GRK5 nuclear accumulation (data not shown), indicating induced GRK5 nuclear translocation also with chronic stimulation of ligands acting through Gq-coupled receptors.

To determine whether the Gq-dependent GRK5 nuclear localization translated into increased cardiomyocyte hypertrophy *in vitro*, NRVMs were infected with AdGRK5 or AdLacZ in the presence of [^{3}H]phenylalanine to investigate active protein synthesis. CAM-Gq expression resulted in myocyte hypertrophy, which was significantly enhanced in the presence of elevated GRK5 (Fig. 3*F*).

Nuclear GRK5 Activates MEF2 *in Vitro*. To determine whether Gq-mediated nuclear translocation and accumulation of GRK5 result in a modification of hypertrophic gene expression in myocytes, we used an MEF2-reporter adenovirus (AdMEF2) and examined activity in the presence of CAM-Gq and GRK5. Although WT GRK5 overexpression resulted in increased MEF2 activity compared with AdLacZ control myocytes, it was not as robust as overexpression of CAM-Gq alone (Fig. 4*A*). However, when AdGRK5 was coinfected with AdCAM-Gq, there was significant enhancement of MEF2 activity (Fig. 4*A*). We have further defined the role of GRK5 in enhancing MEF2 activity in myocytes by showing that this activity depends on catalytic activity and nuclear localization of GRK5, because CAM-Gq + GRK5-K215R (a catalytic inactive mutant) MEF2 activity and CAM-Gq + GRK5-ΔNLS do not significantly increase MEF2 activity over CAM-Gq alone (Fig. 4 *B* and *C*). The above results demonstrate that chronic Gq signaling results in the translocation of GRK5 to the nucleus, where GRK5 activity plays a role in MEF2 activation, which has implications for induction of hypertrophic gene expression.

To address the role of endogenous GRK5 in Gq-mediated regulation of gene transcription, we isolated cardiac fibroblasts from WT and GRK5 KO mice (23, 24) to study MEF2 activity in cells without GRK5. Significant MEF2 activity was seen in both cell types after infection with CAM-Gq over baseline levels (Ad-LacZ–infected control cells); however, there was significantly less Gq-induced MEF2 activity in cells devoid of GRK5 (Fig. 4*D*). Thus, it appears that GRK5 is necessary for maximal activation of Gq-mediated gene transcription via MEF2.

GRK5 Is an HDAC5 Kinase. Previous studies have shown that nuclear MEF2 activation downstream of GPCR-Gq activation involves the phosphorylation of class II HDACs, which are important repressors of MEF2 activity (17, 20, 21, 25). Phosphorylation of class II HDACs leads to their nuclear export, allowing for MEF2 activation and increased hypertrophic gene expression (20, 25). Known HDAC kinases include Ca^{2+}-calmodulin kinase-II (CamKII) and protein kinase D (PKD), but other as yet identified HDAC kinases independent of these two have also been described (20, 21). In myocytes, focus has been on the class II HDAC, HDAC5, which has been shown to be phosphorylated after Gq activation (17, 21). Accordingly, because nuclear GRK5 localization is enhanced after

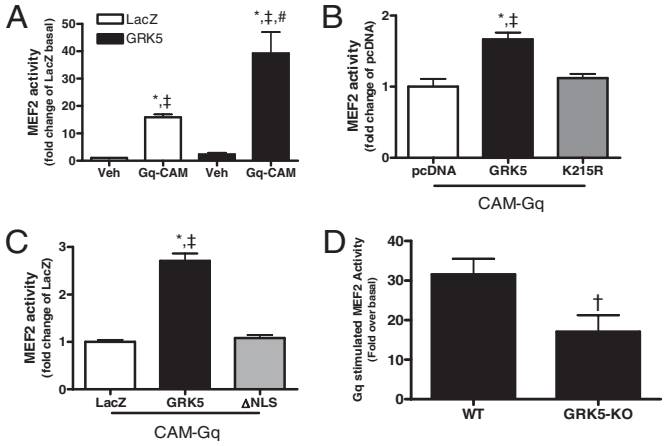

Fig. 4. Gq-mediated MEF2 activity depends on nuclear GRK5 activity. (*A*) NRVMs were infected with a MEF2-luciferase adenovirus and Ad-GRK5 with either Ad-LacZ (control) or Ad-CAM-Gαq. MEF2 activity is normalized to total cellular protein ($n = 4$ separate experiments). *, $P < 0.05$ versus LacZ veh; ‡, $P < 0.05$ versus GRK5 veh; #, $P < 0.05$ versus NLC + CAM-Gq (one-way ANOVA, Bonferroni's multiple comparison test). (*B*) HeLa cells were transfected with pcDNA3.1 (control) and either pcDNA3.1 containing GRK5 or GRK5-K215R (catalytically inactive GRK5) and then infected with Ad-MEF2-luciferase and Ad-CAM-Gαq ($n = 4$ separate experiments). *, $P < 0.05$ versus pcDNA; ‡, $P < 0.05$ versus GRK5-K215R (one-way ANOVA, Bonferroni MCT). (*C*) GRK5 nuclear localization is needed for increased MEF2 activity. NRVMs were infected with Ad-LacZ, Ad-GRK5, or Ad-GRK5ΔNLS (nonnuclear GRK5) and then infected with Ad-MEF2-luciferase and Ad-CAM-Gαq. ($n = 5$ separate experiments). *, $P < 0.05$ versus LacZ; ‡, $P < 0.05$ versus GRK5ΔNLS (one-way ANOVA, Bonferroni MCT). (*D*) Gq-mediated MEF2 activity in cardiac fibroblasts isolated from WT mice and GRK5 KO mice. †$P < 0.05$ Gq-mediated response over basal versus WT cells (Student's *t* test, $n = 5$).

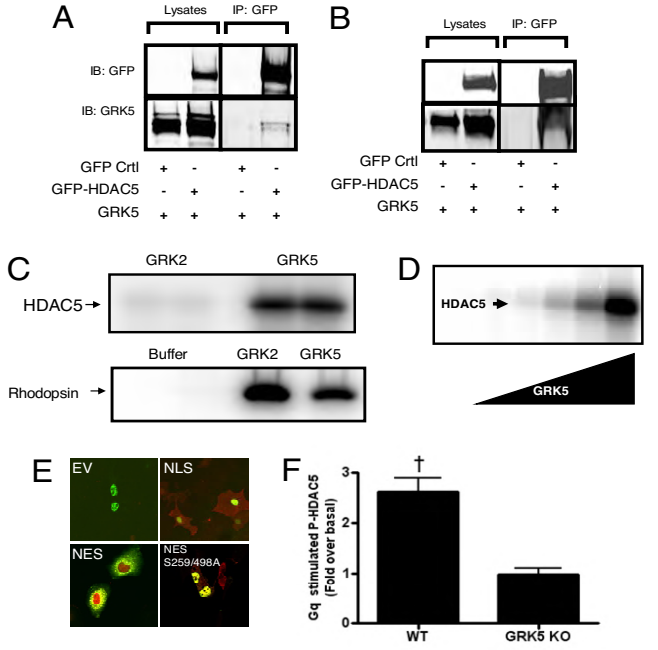

Fig. 5. GRK5 interacts with and phosphorylates HDAC5. (*A* and *B*) NRVMs were infected with adenovirus containing GFP-HDAC5 and GRK5. GFP-HDAC5 was then immunoprecipitated from either whole-cell lysates (*A*) or the nuclear fraction (*B*) and probed for either GFP or GRK5. Cells infected with GRK5 only were used as a negative control. Shown are representative blots from three individual experiments. (*C*) GRK5 but not GRK2 acts as an HDAC kinase on HDAC5. Twenty picomoles of purified GRK5 and GRK2 was mixed with purified GST-HDAC5 (1 μM). Phosphorylation was detected by autoradiography and quantified with ImageQuant ($n = 3$ separate experiments, †$P < 0.05$ two-tailed *t* test). The GRK2 and GRK5 used in these assays were also added to rhodopsin-enriched rod outer segment membranes, and both GRKs were found to be equally active against this GPCR substrate. (*D*) GRK5 is able to phosphorylate GST-HDAC5 in a dose-dependent manner. Increasing picomoles (0.1–20 ρmol) of purified GRK5 resulted in increased HDAC5 phosphorylation. (*E*) Cos-7 cells transfected with plasmids containing GFP-HDAC5 and either (*Upper Left*) empty vector (EV) (*Upper Right*) nuclear-excluded GRK5 and GRK5ΔNLS (in red), or (*Lower Left*) nuclear-trapped GRK5 and GRK5ΔNES (in red). Only cells transfected with the nuclear form of GRK5 (GRK5ΔNES) resulted in specific HDAC5 export. (*Lower Right*) When serines 259 and 498 are mutated to alanines, this nonphosphorylated form of HDAC5 is restricted to the nucleus with coexpression of GRK5ΔNES. (*F*) Endogenous GRK5 is needed for maximal Gq-mediated phosphorylation of HDAC5. Cardiac fibroblasts from either WT or GRK5 KO cells were infected with GFP-HDAC5 and either LacZ (used as basal levels) or CAM-Gαq. After immunoprecipitation of GFP-HDAC5, the phosphorylation status of HDAC5 was detected by using a S498 phosphospecific antibody and normalized to total HDAC5 levels ($P < 0.05$ (*t* test), n = three independent experiments). IB; immunoblot IP; immunoprecipitation.

hypertrophic stimuli and this leads to increased Gq-mediated MEF2 activity, we investigated whether GRK5 might function as an HDAC kinase in the heart.

To test whether GRK5 interacts with HDACs in myocytes, we infected NRVMs with AdGRK5 and with either an empty adenovirus (negative control) or a GFP-tagged HDAC5 adenovirus (AdGFP-HDAC5). Immunoprecipitation of total myocyte lysates with a GFP antibody resulted in the coimmunoprecipitation of GRK5 (Fig. 5*A*). This association between HDAC5 and GRK5 was even more apparent when immunoprecipitation was done with a purified nuclear fraction (Fig. 5*B*). To determine whether this interaction is direct or part of a larger protein complex, purified GRK5 and purified GST-HDAC5 (or GST alone as a control) were incubated together, and after stringent washing, specific and direct binding of GRK5 was found (data not shown). To examine whether GRK5 has any kinase activity toward HDAC5, we used GST-HDAC5 as an *in vitro* substrate and carried out assays with purified GRK5 and GRK2 (Fig. 5*C*). GRK5 was found to robustly phosphorylate HDAC5 whereas GRK2 had no HDAC5 kinase activity (Fig. 5*C*). GRK5 was able to phosphorylate HDAC5 in a dose-dependent manner (Fig. 5*D*). GRK5 kinase activity was found to be on at least one known HDAC kinase site, Ser-259, because incubation with GRK5 and the Ser-259→Ala mutant GST-HDAC5 resulted in negligible phosphorylation (data not shown).

To determine whether this interaction with, and phosphorylation of, HDAC5 by GRK5 can occur in the nuclei of intact cells, we examined HDAC5 nuclear export in cells where GRK5 was targeted and enriched in the nucleus (Fig. 5*E*). Expression of the nuclear-excluded GRK5ΔNLS in COS-7 cells did not alter the nuclear localization of GFP-HDAC5 (Fig. 5*E Upper Right*). When the nuclear export sequence [NES, residues 259–265 (8)] of GRK5 was mutated (GRK5ΔNES), resulting in a GRK5 with increased nuclear localization, GFP-HDAC5 was actively exported from the nucleus (Fig. 5*E Lower Left*). When GRK5-ΔNES was coexpressed in cells with a GFP-HDAC5 mutant where the two known HDAC kinase sites, serine residues 259 and 498, were changed to alanine, nuclear GRK5 activity failed to direct the export of this phosphorylation-resistant HDAC5 (Fig. 5*E Lower Right*). Thus, it appears that a nuclear interaction takes place in the intact cell leading to HDAC5 export, which is known to depend on phosphorylation.

We next investigated the role of endogenous GRK5 in the phosphorylation of HDAC5 by using a S498 phosphospecific HDAC5 antibody. We took WT and GRK5 KO cardiac fibroblasts and expressed either LacZ as a control (basal) or CAM-Gαq (Fig. 5*F*). After immunoprecipitation of HDAC5, there was significantly more HDAC5 phosphorylation, normalized to total HDAC5, after Gq activation in WT cardiac fibroblasts compared with the GRK5 KO fibroblasts (Fig. 5*F*). From these data, we are able to conclude that endogenous GRK5 appears to be a key HDAC5 kinase downstream of Gq activation.

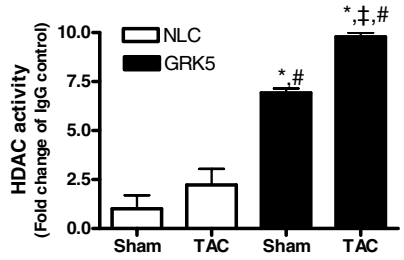

Fig. 6. GRK5 interacts with HDACs *in vivo*. GRK5 was immunoprecipitated from TgGRK5 or NLC hearts under both sham and 2 weeks post-TAC. After IP, HDAC activity was measured by using the Caymen HDAC activity assay (see Methods). (n = 3–5 mice per group). *, $P < 0.05$ versus NLC sham; ‡, $P < 0.05$ versus GRK5 sham, #, $P < 0.05$ versus NLC post-TAC (one-way ANOVA, Bonferroni's multiple comparison test). IB, immunoblot.

GRK5 Interacts with HDACs *in Vivo*. It is obviously critical to translate the *in vitro* findings of GRK5 being an HDAC kinase *in vivo*. To do this, we determined whether GRK5 can interact with HDACs in the intact mouse heart. We immunoprecipitated GRK5 from NLC and Tg-GRK5 hearts under sham conditions and after 2 weeks of TAC (Fig. 6). We found significant HDAC activity associated with GRK5 over an IgG control, and TAC increased GRK5-associated HDAC activity, which was higher with GRK5 overexpression (Fig. 6), supporting the above finding of increased nuclear accumulation of GRK5 after TAC. To demonstrate that this *in vivo* HDAC activity was specific for GRK5, we immunoprecipitated overexpressed GRK2 from TgGRK2 hearts and found no appreciable HDAC activity (data not shown). In separate experiments with TgGRK5 and TgGRK5ΔNLS mice, we found no significant increase in cytosolic HDAC activity associated with GRK5 in TgGRK5ΔNLS hearts (data not shown), supporting the lack of nuclear activity *in vivo* of this mutant GRK5.

Discussion

To date, the principal actions of GRKs have been ascribed to their role on desensitizing seven-transmembrane–spanning GPCRs and inducing β-arrestin recruitment to the phosphorylated receptor (1). We have uncovered data in this study demonstrating that GRKs may have multiple functions and substrates based on their cellular localization, and we demonstrate an *in vivo* physiological relevance of a non-GPCR substrate for the GRK, GRK5. Our current data show that GRK5 can act in a previously unappreciated manner in the heart to directly modulate nuclear events downstream of Gq-mediated hypertrophic signals. In addition, we have found that GRK5 can act as a class II HDAC kinase, apparently targeting HDAC5 in the nuclei of cardiomyocytes in a manner similar to other known HDAC kinases such as PKD, with associated regulation of MEF2-mediated gene transcription. As a consequence of this HDAC kinase activity, we show that when GRK5 is elevated in the nucleus of cardiomyocytes, it can lead to a pathological phenotype after pressure-overload stress, which is clinically relevant because GRK5 is elevated in human HF (2). Thus, the nuclear activity of enhanced GRK5 could contribute to HF progression through maladaptive cardiac growth.

Because overexpression of a nuclear-deficient GRK5 mutant does not lead to the *in vivo* post-TAC pathological phenotype seen in WT GRK5 overexpressors, its membrane GPCR-dependent activity can be viewed as separate from its nuclear activity. This is important, because it has recently been suggested that GRK5 actions on the membrane-embedded α_1-AR can be protective to the heart during certain stress conditions through the β-arrestin–dependent transactivation of the EGF receptor (6). Our data show that when GRK5 is elevated in the heart, any such protective mechanism or any other GPCR-dependent event after pressure-overload stress is not enough to overcome the pathological signaling induced by nuclear GRK5 activity that appears to be on class II HDACs, leading to maladaptive gene expression. However, these protective signaling mechanisms may still be active.

Histone deacetylases are a group of proteins involved in DNA regulation through the deacetylation of histones, resulting in chromosome condensation and transcriptional repression (16). Within the heart under basal conditions, the class II HDACs bind and inhibit the transcription factor MEF2 (25, 26), repressing hypertrophic genes associated with the fetal gene program. Hypertrophic stimuli, including agents acting through GPCRs, set in motion a series of protein kinase cascades in which HDACs are phosphorylated, separated from MEF2, and transported out of the nucleus, allowing MEF2-mediated gene activation and associated hypertrophy (25, 26). Histone deacetylase 5 appears to be especially important in the heart at regulating MEF2 activity and hypertrophic growth (21). Because HDAC phosphorylation is an essential step in the activation of MEF2-associated hypertrophic genes, HDAC kinase inhibition appears to be an attractive therapeutic target.

Known HDAC kinases include PKD and CamKII, and now it appears that GRK5 can be added to this list, because our data show that nuclear accumulation of GRK5 in response to chronic Gq signaling results in increased MEF2 activity *in vitro* and significantly exaggerated *in vivo* expression of atrial natriuretic factor and β-myosin heavy chain expression, two fetal genes associated with the hypertrophic phenotype. In addition, *in vitro* hypertrophic experiments in myocytes show that increased MEF2 activity seen with elevated nuclear GRK5 translated to an increase in active protein synthesis and increased cell size. Importantly, this increased MEF2 activity was found to depend both on the nuclear localization of GRK5 and its catalytic activity. To further support GRK5 being an HDAC kinase *in vivo* are the findings that HDAC5 and HDAC9 KO mice present a phenotype that enhances cardiac hypertrophy after stress (25). This is similar to what we see in TgGRK5 mice after TAC, which would be expected with enhanced HDAC kinase activity, because both the HDAC KO models and enhanced HDAC kinase activity results in the loss of HDAC in the nucleus to repress hypertrophic gene expression. In cells devoid of GRK5, we found less Gq-mediated MEF2 activation and phosphorylated HDAC5, demonstrating that endogenous GRK5 is part of the downstream signaling events leading to Gq-mediated myocyte hypertrophy. We have previously shown the Gq is the final common trigger for pressure-overload ventricular hypertrophy (19) and, thus, our results would appear to have physiological significance that is even more critical in disease states such as HF, where GRK5 is elevated in myocytes. Therefore, GRK5 can be an important HDAC kinase that may be targeted for prevention of maladaptive hypertrophy.

GRK5, like the other GRKs, is highly regulated through expression levels, subcellular localization, and activity (1, 14, 27). Concerning our current results showing nuclear regulation of GRK5 downstream of Gq activation, it is important to note that GRK5 has been shown to be regulated by molecules that would be expected to be present in Gq-signaling cascades. This includes data showing that PKC is able to phosphorylate GRK5, resulting in autophosphorylation and inhibition of GPCR substrate affinity (28). Further calcium/calmodulin can bind to GRK5 with higher affinity than other GRKs, inhibiting its activity toward known substrates (29). GRK5 is also able to bind phosphatidylinositol 4,5-bisphosphate within its amino terminus, allowing for GRK5 to properly localize on the membrane (30), whereas binding of GRK5 to the plasma membrane through its carboxyl terminus causes autophosphorylation and increases its kinase activity toward GPCRs (31). How this GRK5 regulation directs the HDAC activity of GRK5 remains to be determined.

The nuclear localization of GRK5 is not a new finding as it has been recently shown to have a functional NLS along with other GRK4-like GRK subfamily members (8, 32), and, in fact, nuclear accumulation has been demonstrated in myocytes of hypertensive rats (9). However, the functional role of nuclear GRK5 is not

known, and, interestingly, this kinase also appears to have direct DNA binding capabilities (8). Of note, previous data show that calmodulin binding to GRK5 within its carboxyl terminus results not only in autophosphorylation and inhibition of GPCR kinase activity (28, 33) but in its nuclear export in some cells (8).

One question that arises from these data is how the HDAC kinase activity of GRK5 aligns with or affects other known HDAC kinases such as PKD and CamKII. Multiple HDAC kinases downstream of hypertrophic signals in the heart could potentially serve as redundant pathways of regulation that may become pathological due to changes in kinase levels and subcellular localization. This appears to be the case for GRK5, which is elevated in compromised myocardium, and thus could cause the structure of the failing heart to become prone to maladaptation. Indeed, this is supported by our present post-TAC phenotypes in TgGRK5 mice versus TgΔNLS mice, where the nuclear localization of GRK5 appears to be responsible for exaggerated hypertrophy and early HF. Overall, it appears that G protein activation (at least for Gq) can induce GRK5's GPCR desensitization activity (5) and nuclear accumulation and HDAC kinase activity. Moreover, our data show that GRK5 nuclear accumulation and direct regulation of gene expression in response to hypertrophic stimuli represent a role for GRK5 that goes beyond the classically established role of GRK5 in GPCR regulation and desensitization.

Materials and Methods

Immunoprecipitation and Western Blot Analysis, Cellular Fractionations Immunofluorescence, and Echocardiography. For details, see *SI Materials and Methods* and Table S2.

GST-HDAC Expression, Purification, and Phosphorylation and HDAC Activity Assay. GST or GST-HDAC5 fusion protein expression and HDAC activity assay methods are described in detail in *SI Materials and Methods*.

Luciferase Assays. Cells were harvested 24–48 h after transfection/infection in passive lysis buffer (Promega). The samples were prepared according to the manufacturer's protocol (Promega) and measured by using a Victor plate reader. Luciferase units were normalized to total protein.

***In Vitro* Cellular Hypertrophy (Protein Synthesis), Isolation of Cardiac Myocytes and Maintenance of Primary Cultures, RNA Isolation and Semiquantitative PCR, and TAC.** See *SI Materials and Methods* for detailed cellular hypertrophy assay methods, detailed isolation methods for NRVMs and adult cardiomyocytes, and detailed RT-PCR methods. TAC was performed essentially as described previously (19), but see *SI Materials and Methods* for detailed TAC surgical methods.

Statistics. All values in the text and figures are presented as mean ± SEM from at least three independent experiments from given n sizes. Statistical significance of multiple treatments was determined by one-way ANOVA followed by the Bonferroni post hoc test when appropriate. Statistical significance between two groups was determined by using the two-tailed Student's t test. P values <0.05 were considered significant.

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1. Premont RT, Gainetdinov RR (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol* 69:511–534.
2. Dzimiri N, Muiya P, Andres E, Al-Halees Z (2004) Differential functional expression of human myocardial G protein receptor kinases in left ventricular cardiac diseases. *Eur J Pharmacol* 489:167–177.
3. Rockman HA, Koch WJ, Lefkowitz RJ (2002) Seven-transmembrane-spanning receptors and heart function. *Nature* 415:206–212.
4. Rockman HA, *et al.* (1996) Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. *Proc Natl Acad Sci USA* 93:9954–9959.
5. Eckhart AD, *et al.* (2000) Hybrid transgenic mice reveal in vivo specificity of G protein-coupled receptor kinases in the heart. *Circ Res* 86:43–50.
6. Noma T, *et al.* (2007) Beta-arrestin–mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest* 117:2445–2458.
7. Iaccarino G, Lefkowitz RJ, Koch WJ (1999) Myocardial G protein-coupled receptor kinases: Implications for heart failure therapy. *Proc Assoc Am Physicians* 111:399–405.
8. Johnson LR, Scott MG, Pitcher JA (2004) G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. *Mol Cell Biol* 24:10169–10179.
9. Yi XP, Gerdes AM, Li F (2002) Myocyte redistribution of GRK2 and GRK5 in hypertensive, heart-failure-prone rats. *Hypertension* 39:1058–1063.
10. Freeman JL, De La Cruz EM, Pollard TD, Lefkowitz RJ, Pitcher JA (1998) Regulation of G protein-coupled receptor kinase 5 (GRK5) by actin. *J Biol Chem* 273:20653–20657.
11. Pronin AN, Morris AJ, Surguchov A, Benovic JL (2000) Synucleins are a novel class of substrates for G protein-coupled receptor kinases. *J Biol Chem* 275:26515–26522.
12. Carman CV, Lisanti MP, Benovic JL (1999) Regulation of G protein-coupled receptor kinases by caveolin. *J Biol Chem* 274:8858–8864.
13. Carman CV, Som T, Kim CM, Benovic JL (1998) Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. *J Biol Chem* 273:20308–20316.
14. Ribas C, *et al.* (2007) The G protein-coupled receptor kinase (GRK) interactome: Role of GRKs in GPCR regulation and signaling. *Biochim Biophys Acta* 1768:913–922.
15. Koch WJ, *et al.* (1995) Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. *Science* 268:1350–1353.
16. McKinsey TA, Zhang CL, Olson EN (2001) Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev* 11:497–504.
17. Liu N, Olson EN (2006) Coactivator control of cardiovascular growth and remodeling. *Curr Opin Cell Biol* 18:715–722.
18. Dorn GW, 2nd, Force T (2005) Protein kinase cascades in the regulation of cardiac hypertrophy. *J Clin Invest* 115:527–537.
19. Akhter SA, *et al.* (1998) Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science* 280:574–577.
20. McKinsey TA (2007) Derepression of pathological cardiac genes by members of the CaM kinase superfamily. *Cardiovasc Res* 73:667–677.
21. Backs J, Olson EN (2006) Control of cardiac growth by histone acetylation/deacetylation. *Circ Res* 98:15–24.
22. Adams JW, *et al.* (1998) Enhanced Galphaq signaling: A common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc Natl Acad Sci USA* 95:10140–10145.
23. Gainetdinov RR, Premont RT, Caron MG, Lefkowitz RJ (2000) Reply: Receptor specificity of G-protein-coupled receptor kinases. *Trends Pharmacol Sci* 21:366–367.
24. Gainetdinov RR, *et al.* (1999) Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. *Neuron* 24:1029–1036.
25. Zhang CL, *et al.* (2002) EN Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell* 110:479–488.
26. Chang S, *et al.* (2004) Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. *Mol Cell Biol* 24:8467–8476.
27. Penn RB, Pronin AN, Benovic JL (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc Med.* 10:81–89.
28. Pronin AN, Benovic JL (1997) Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J Biol Chem* 272:3806–3812.
29. Pronin AN, Satpaev DK, Slepak VZ, Benovic JL (1997) Regulation of G protein-coupled receptor kinases by calmodulin and localization of the calmodulin binding domain. *J Biol Chem* 272:18273–18280.
30. Pitcher JA, *et al.* (1996) Phosphatidylinositol 4,5-bisphosphate (PIP2)-enhanced G protein-coupled receptor kinase (GRK) activity. Location, structure, and regulation of the PIP2 binding site distinguishes the GRK subfamilies. *J Biol Chem* 271:24907–24913.
31. Kunapuli P, Gurevich VV, Benovic JL (1994) Phospholipid-stimulated autophosphorylation activates the G protein-coupled receptor kinase GRK5. *J Biol Chem* 269:10209–10212.
32. Jiang X, Benovic JL, Wedegaertner PB (2007) Plasma membrane and nuclear localization of G protein coupled receptor kinase 6A. *Mol Biol Cell* 18:2960–2969.
33. Pronin AN, Carman CV, Benovic JL (1998) Structure-function analysis of G protein-coupled receptor kinase-5. Role of the carboxyl terminus in kinase regulation. *J Biol Chem* 273:31510–31518.