Activated glycogen synthase-3β suppresses cardiac hypertrophy *in vivo*

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The adult myocardium responds to a variety of pathologic stimuli by hypertrophic growth that frequently progresses to heart failure. The calcium/calmodulin-dependent protein phosphatase calcineurin is a potent transducer of hypertrophic stimuli. Calcineurin dephosphorylates members of the nuclear factor of activated T cell (NFAT) family of transcription factors, which results in their translocation to the nucleus and activation of calcium-dependent genes. Glycogen synthase kinase-3 (GSK-3) phosphorylates NFAT proteins and antagonizes the actions of calcineurin by stimulating NFAT nuclear export. To determine whether activated GSK-3 can act as an antagonist of hypertrophic signaling in the adult heart *in vivo*, we generated transgenic mice that express a constitutively active form of GSK-3β under control of a cardiac-specific promoter. These mice were physiologically normal under nonstressed conditions, but their ability to mount a hypertrophic response to calcineurin activation was severely impaired. Similarly, cardiac-specific expression of activated GSK-3β diminished hypertrophy in response to chronic β-adrenergic stimulation and pressure overload. These findings reveal a role for GSK-3β as an inhibitor of hypertrophic signaling in the intact myocardium and suggest that elevation of cardiac GSK-3β activity may provide clinical benefit in the treatment of pathologic hypertrophy and heart failure.

The adult myocardium undergoes hypertrophic growth in response to a variety of pathologic stimuli, including myocardial infarction, valvular disease, hypertension, endocrine disorders, and inherited mutations in components of the cytoskeleton and sarcomere (reviewed in refs. 1 and 2). Prolonged hypertrophy is correlated with poor clinical prognosis (3) and frequently progresses to dilated cardiomyopathy and sudden death. There is a critical need to identify novel drug targets that can be exploited to mitigate the adverse sequelae of hypertrophy.

Several calcium-dependent signal transduction pathways have been implicated in cardiac hypertrophy and heart failure (reviewed in refs. 4 and 5). The calcium/calmodulin-dependent protein phosphatase calcineurin is an especially potent inducer of myocardial hypertrophy *in vivo* and *in vitro* (6). Numerous studies have demonstrated that calcineurin activation is required for hypertrophy, as shown by the ability of the calcineurin inhibitors, cyclosporin A and FK-506, to prevent or diminish cardiac growth in response to pressure overload, hypertension, myocardial infarction, and contractile abnormalities (reviewed in refs. 7–9). Further evidence for the requisite role of calcineurin in hypertrophy has come from the findings that cardiac-specific over-expression of the calcineurin-inhibitory proteins, myocyte-enriched calcineurin-interacting protein (MCIP), and Cabin/Cain can prevent hypertrophy not only due to constitutive calcineurin activation in transgenic mice, but also due to aortic constriction (10, 11).

Calcineurin dephosphorylates a variety of cellular targets that could, in principle, influence cardiac growth and function. Among these are nuclear factor of activated T cell (NFAT) proteins, a family of calcium-regulated transcription factors with diverse roles in development and disease (12). NFAT proteins contain an N-terminal regulatory domain that controls their nuclear import in response to calcineurin activation. In unstimulated cells, this regulatory domain is phosphorylated and masks the nuclear localization sequence, causing NFAT proteins to be sequestered in the cytoplasm (13). Calcineurin dephosphorylates the NFAT regulatory domain, inducing a conformational change in the protein that promotes nuclear import (13). Maintenance of NFAT proteins in the nucleus requires continuous calcineurin signaling and is counteracted by several nuclear export kinases that phosphorylate the calcineurin substrate sites in the N-terminal regulatory domain (13–15).

Glycogen synthase kinase-3 (GSK-3) is a ubiquitous serine/threonine protein kinase that phosphorylates a series of sites in the N-terminal regulatory regions of NFAT proteins following the action of one or more priming kinases (13). In contrast to other kinases, GSK-3 is highly active in unstimulated cells and becomes inactivated in response to mitogenic stimulation (reviewed in ref. 16). The activity of GSK-3 is controlled by the phosphorylation status of serine-9. Phosphorylation of this site inhibits GSK-3 activity by creating an inhibitory pseudosubstrate for the enzyme (17). Conversely, dephosphorylation of this site, or mutations that prevent phosphorylation, result in activation of the kinase. Several protein kinases, including Akt/protein kinase B (PKB), have been shown to phosphorylate serine-9 of GSK-3 in response to mitogenic signaling, thereby inactivating the enzyme (18). GSK-3 is also inhibited by Wingless/Wnt signaling through a mechanism independent of serine-9 phosphorylation (reviewed in ref. 19).

We showed previously that a mutant form of NFATc4 (NFAT3) lacking the N-terminal regulatory region and GSK-3 phosphorylation sites was localized constitutively to the nucleus and induced hypertrophy independent of upstream signaling by calcium and calcineurin (6). The ability of GSK-3 to inactivate NFAT proteins by preventing their accumulation in the nucleus suggests that GSK-3 might possess antihypertrophic activity. Indeed, activated GSK-3 was recently shown to diminish the responsiveness of primary neonatal cardiomyocytes to hypertrophic stimuli (20, 21). Serine-9 of GSK-3 has also been shown to be phosphorylated in failing human hearts (22), which would be predicted to inactivate the kinase and thereby augment the prohypertrophic activity of calcineurin.

In this study, we investigated whether activation of GSK-3β was sufficient to prevent cardiac hypertrophy *in vivo*. Our results show that cardiac-specific expression of a signal-resistant form of GSK-3β, containing a serine-9 to alanine mutation, diminishes the hypertrophic growth response to calcineurin activation,

Abbreviations: ANF, atrial natriuretic factor; BNP, brain-type natriuretic protein; GSK, glycogen synthase kinase; MHC, myosin heavy chain; NFAT, nuclear factor of activated T cells; PI3-K, phosphoinositide 3-kinase.

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adrenergic stimulation, and pressure overload. These findings suggest that measures that enhance cardiac GSK-3β activity, directly or indirectly, could be clinically beneficial in the treatment of cardiac hypertrophy and heart failure.

Materials and Methods

Generation of Transgenic Mice. A cDNA encoding mouse hemagglutinin-taggged GSK-3β with a serine-to-alanine mutation at position 9 was cloned into a cardiac-specific expression plasmid containing the α-myosin heavy chain (MHC) promoter and human GH (hGH) poly(A)$^+$ signal (23). DNA isolation and oocyte injections were performed as described by using oocytes derived from B6C3 mice (6). Genomic DNA was isolated from mouse tail biopsies and was analyzed by Southern blotting with a probe specific for the hGH poly(A)$^+$ signal. Mice were obtained from the National Cancer Institute (Frederick, MD) and animal handling was performed according to University of Texas Southwestern institutional guidelines.

Isoproterenol Administration. Isoproterenol was delivered chronically to mice using a surgically implanted miniosmotic pump [Durect (Cupertino, CA) model 1002] that released isoproterenol in 0.9% NaCl at a rate of 30 μg per gram of body weight per hour. Control pumps delivered 0.9% NaCl solution alone. Nine days after implantation of pumps, hearts were harvested and the heart weight/body weight ratios were determined and compared between control and isoproterenol-treated animals.

RNA Isolation and Analysis. Total RNA was prepared from mouse hearts by using Trizol Reagent (Roche Molecular Biochemicals). Atrial natriuretic factor (ANF) and brain-type natriuretic protein (BNP) mRNA expression was assessed by Northern blot analysis using radiolabeled probes derived from the rat ANF and mouse BNP cDNAs and 10 μg of total RNA. Membranes were rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to normalize for differences in RNA loading. RNA expression levels were quantified using a Molecular Dynamics Storm PhosphorImager. β-MHC mRNA expression was determined by semiquantitative reverse transcription (RT)-PCR using the following primers: [+], 5′-tgcaaaggctccaggtctgagggc-3′; [−], 5′-gccaacaccaacctgtccaagttc-3′. Reactions were carried out in the presence of [α-^{32}P]dCTP and products were resolved through 5% polyacrylamide gels. To account for differences in cDNA input, parallel reactions were performed using GAPDH-specific primers ([+], 5′-gccatcaacgacccttcattg-3′; [−], 5′-actccacgacatactcagcacc-3′). Radiolabeled products were quantified using a PhosphorImager.

Western Blot Analysis. Protein extracts were prepared by homogenization of frozen mouse hearts in 62.5 mM Tris (pH6.8) containing 2% wt/vol SDS, 10% glycerol, and 50 mM DTT. Proteins were resolved by SDS/PAGE using 8% gels and transferred to PVDF membranes (Sequi-Blot, Bio-Rad). Immunoblotting was performed using mouse anti-GSK-3 monoclonal (0011-A, Santa Cruz Biotechnology) and rabbit anti-hemagglutinin polyclonal (Y11, Santa Cruz Biotechnology) antibodies at dilutions of 1:500 and 1:1000, respectively.

Histology. For histological analysis, excised hearts were rinsed in PBS and incubated in Krebs-Hanseleit solution lacking Ca^{2+} to relax the cardiac muscle before fixation. Hearts were fixed in 10% formalin for 12 h at 4°C. Samples were dehydrated with ethanol, mounted in paraffin, and sectioned at 5-μm thickness. Sections were stained either with hematoxylin and eosin (H&E) to visualize tissue architecture, Masson's trichrome stain to detect collagen deposits, or used for immunofluorescence. The cross-sectional area of ventricular papillary muscles was determined using SCION IMAGE 1.62C acquisition and analysis software (Scion, Frederick, MD).

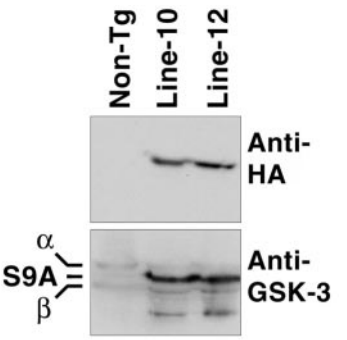

Fig. 1. α-MHC-GSK-3βS9A transgene expression. Heart extracts were prepared from transgenic and nontransgenic littermates and analyzed for expression of GSK-3 by Western blot analysis as described in *Materials and Methods*. (*Upper*) Anti-hemagglutinin antibody detects the epitope-tagged GSK-3βS9A protein in extracts from transgenic lines 10 and 12. (*Lower*) Anti-GSK-3 antibody detects endogenous GSK-3 isoforms, as well as exogenous GSK-3S9A. The GSK-3βS9A protein is expressed at a level ≈8-fold higher than endogenous α and β isoforms.

Immunofluorescence. Frontal plane paraffin sections were mounted on silanated microscope slides and heated to 54°C for 40 min. Slides were deparaffinized with xylene and rehydrated in graded ethanol solutions until equilibrated in PBS with 0.1% Tween-20 (PBST). Sections were microwaved in citrate buffer (BioGenex; pH 6.0) to facilitate retrieval of NFATc1 antigenic epitopes and washed twice with 100 mM glycine/PBST to quench autofluorescence. Sections were blocked for 1 h with IgG blocking reagent (M.O.M. Kit, Vector Laboratories), washed in PBST, and equilibrated in M.O.M. kit diluent. Sections were then incubated with NFATc1 (7A6) antibody (a gift from G. Crabtree, Stanford University, Stanford, CA) at a dilution of 1:300 in M.O.M. diluent for 30 min at room temperature. Sections were stained with biotinylated anti-mouse IgG reagent (M.O.M., Vector Laboratories) for 10 min, then with fluorescein-conjugated avidin DCS (Vector Laboratories) for 5 min, and then coverslipped in Vectashield (Vector Laboratories).

Thoracic Banding of Aorta. Wild-type and transgenic littermates underwent either a sham operation or were subjected to pressure overload by thoracic aorta banding as described (24).

Results

Generation of α-MHC-GSK-3βS9A Transgenic Mice. To investigate the potential role of GSK-3β as a negative regulator of hypertrophic signaling *in vivo*, we generated transgenic mice that expressed a constitutively active form of GSK-3β (GSK-3βS9A) under control of the α-MHC promoter. The constitutively active form of GSK-3β contained a serine-9 to alanine mutation, which prevents inactivation of the enzyme by Akt phosphorylation (25).

Two independent mouse lines were obtained with the α-MHC-GSK-3βS9A transgene. Expression of the exogenous GSK-3 protein was confirmed by Western blot analysis using anti-hemagglutinin antibody (Fig. 1 *Upper*). Exogenous GSK-3β was expressed at significantly higher levels than the endogenous protein, as determined by immunoblotting with an anti-GSK antibody (Fig. 1 *Lower*). Transgenic mice appeared normal, but exhibited a slight (9%) but statistically significant reduction in heart weight/body weight ratios (Fig. 2 *A* and *B*). This reduction in heart size had no apparent effect on cardiac function or longevity.

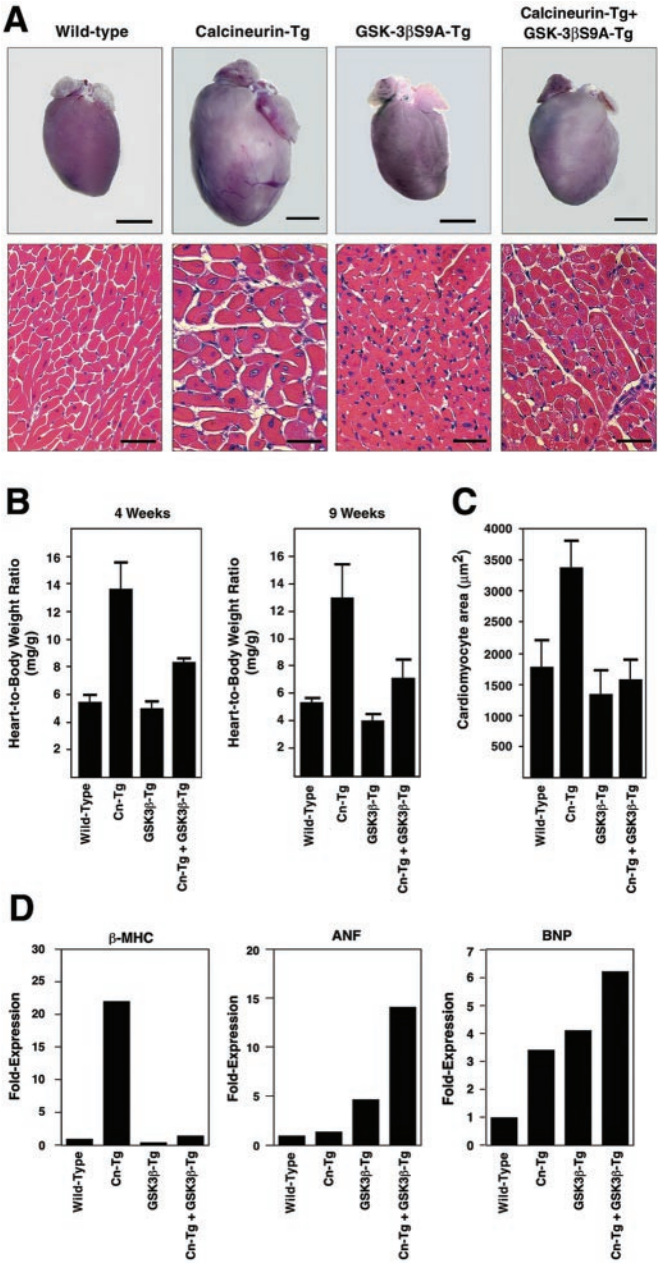

Fig. 2. Effects of GSK-3βS9A on cardiac hypertrophy induced by activated calcineurin. (*A*) Hearts were removed from 4-week-old mice of the indicated genotypes. (Scale bar, 2 mm.) Histological sections stained with hematoxylin and eosin (H&E) are shown at $\times$40 magnification. (Scale bar, 0.02 mm.) (*B*) Heart-to-body-weight ratios of 4- and 9-week-old mice of the indicated genotypes were determined. Heart sizes of at least six mice of each genotype were determined. Values represent mean $\pm$ SD. (*C*) Cardiomyocyte area was determined in histological sections from 4-week-old mice shown in *A*, as described in *Materials and Methods*. Values represent mean $\pm$ SD. (*D*) Expression of transcripts for β-MHC, ANF, and BNP in RNA samples from 4-week-old mice of the indicated genotypes were determined as described in *Materials and Methods*.

Activated GSK-3β Inhibits Cardiac Hypertrophy in Response to Activated Calcineurin. Our primary goal was to determine whether activated GSK-3β could interfere with hypertrophic signaling in the heart. Mice harboring an α-MHC-calcineurin transgene that directs the expression of a constitutively active form of calcineurin in the heart develop massive cardiac hypertrophy by 4 weeks of age (Fig. 2*A* and ref. 6). Coexpression of GSK-3βS9A with activated calcineurin resulted in a significant reduction in cardiac size (Fig. 2*A*). At 4 weeks of age, the calcineurin transgene evoked a 150% increase in cardiac mass, which was reduced to a 59% increase ($P < 0.001$) in the presence of the GSK-3βS9A transgene (Fig. 2*B*). Similar heart proportions were observed in 9-week-old animals (Fig. 2*B*). Morphometric analysis of the cross-sectional area of cardiomyocytes from histological sections showed that activated calcineurin resulted in a 70% increase in myocardial cell area; this effect of calcineurin was abolished by GSK-3βS9A (Fig. 2*C*). GSK-3βS9A expression also extended the lifespan and delayed the progression to heart failure of α-MHC-calcineurin transgenic mice (data not shown).

Cardiac hypertrophy is accompanied by activation of a set of fetal cardiac genes, including those encoding β-MHC, ANF, and BNP. At 4 weeks of age, β-MHC expression was up-regulated over 20-fold in the hearts of α-MHC-calcineurin transgenic mice, and this increase was prevented by GSK-3βS9A (Fig. 2*D*), consistent with its antihypertrophic effects. Paradoxically, however, GSK-3S9A alone stimulated expression of ANF and BNP and it potentiated the effects of calcineurin. These findings demonstrate that the regulation of β-MHC can be uncoupled from that of ANF and BNP and that expression of these fetal genes does not directly correlate with hypertrophy.

Activated GSK-3β Inhibits Cardiac Hypertrophy in Response to Adrenergic Stimulation and Pressure Overload. To test whether activated GSK-3β could prevent hypertrophy evoked by β-adrenergic stimulation, we used implantable, osmotic minipumps to chronically administer isoproterenol over a 9-day period. This procedure resulted in a 50% increase in cardiac mass in wild-type animals compared with saline control littermates (Fig. 3 *A* and *B*). This response was blunted in GSK-3βS9A transgenic mice ($P < 0.01$). Histological sections showed that the reduction in cardiac size in GSK-3βS9A transgenic mice was due to a lack of ventricular hypertrophy. In contrast, isoproterenol treatment caused similar enlargement of the atria in GSK-3S9A transgenic and nontransgenic mice, suggesting that its effects were restricted primarily to the ventricles (Fig. 3*A*). GSK-3βS9A also reduced fibrosis in response to isoproterenol administration.

To determine whether GSK-3β could antagonize the hypertrophic response to physiologically relevant pressure overload, mice were subjected to surgical constriction of the thoracic aorta. Pressure overload was tolerated equally well by transgenic and wild-type littermates with no signs of cardiovascular compromise or increased mortality. Left ventricular mass normalized to tibia length increased 64% ($P < 0.01$) in banded wild-type mice. In contrast, hypertrophic growth was significantly ($P < 0.01$) blunted in GSK-3β transgenic mice (increased 24%) (Fig. 3*C*). Despite the imposition of additional 50 mmHg systolic pressure by aortic banding, abolition of the hypertrophic response by GSK-3βS9A did not result in left ventricular decompensation.

Expression of ANF and BNP was enhanced by GSK-3βS9A in response to adrenergic stimulation and pressure overload, analogous to the response to calcineurin activation (data not shown).

Activated GSK-3β Reduces Nuclear Localization of NFAT. To determine whether activated GSK-3β prevented nuclear accumulation of NFAT in the presence of activated calcineurin, we examined the subcellular distribution of NFATc1 in ventricular cardiomyocytes from transgenic mice (Fig. 4). No NFAT nuclear staining was detected in cardiomyocytes from nontransgenic (wild-type) hearts. In contrast, punctate intranuclear staining of NFATc1 was readily detected in histological sections from α-MHC-calcineurin transgenic mice (Fig. 4*A*). In calcineurin/GSK-3βS9A double transgenics, there was an approximate 2-fold decrease ($P < 0.001$) in the number of cardiomyocytes with nuclear NFAT immunostaining (Fig. 4*B*). However, nuclear NFAT immunostaining was clearly detected in the presence of

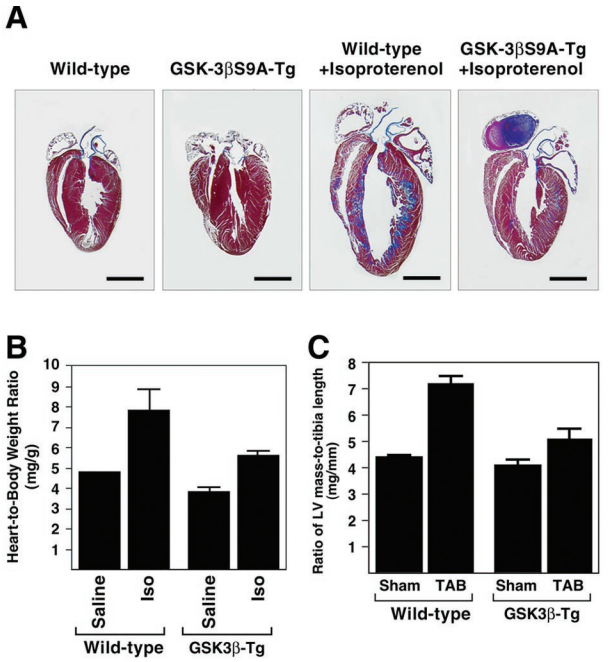

Fig. 3. Effects of GSK-3βS9A on cardiac hypertrophy induced by isoproterenol stimulation and pressure overload. (*A*) Masson-trichrome staining of hematoxylin and eosin (H&E)-stained histological sections of hearts from animals implanted with miniosmotic pumps that delivered saline or isoproterenol. (Scale bar, 2 mm.) Note that the heart from the GSK-3βS9A transgenic treated with isoproterenol developed an atrial thrombosis. (*B*) Heart-to-body-weight ratios of mice of the indicated genotypes were determined. Heart sizes of at least six mice of each genotype were determined. (*C*) The ratio of LV mass to tibia length of mice of the indicated genotypes were determined 21 days after thoracic aortic banding (TAB). Sham-operated littermates served as controls. At least five mice of each genotype were analyzed. Values represent mean ± SD.

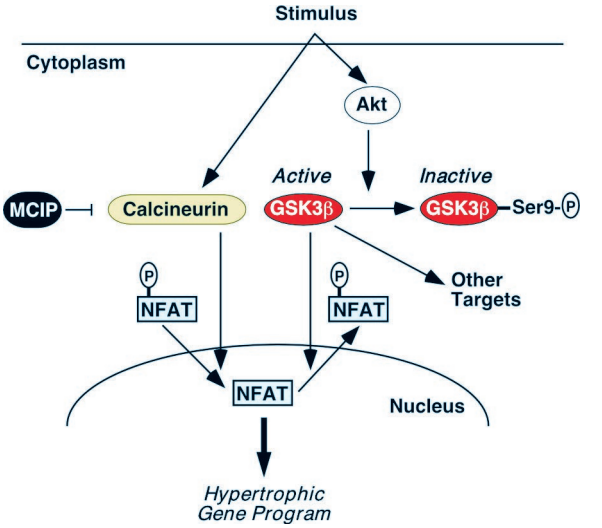

Fig. 5. A model depicting the antagonistic effects of calcineurin and GSK-3 on NFAT activity and cardiac hypertrophy. Numerous hypertrophic stimuli activate calcineurin and Akt. Calcineurin dephosphorylates NFAT proteins, which results in their translocation into the nucleus and activation of the hypertrophic gene program. GSK-3β antagonizes calcineurin signaling by phosphorylation NFAT proteins. GSK-3β is inactivated by phosphorylation of serine-9 by Akt and other kinases. GSK-3β also has other substrates that could influence hypertrophic signaling. Calcineurin activity is also inhibited by myocyte-enriched calcineurin-interacting protein (MCIP).

calcineurin and GSK-3βS9A, indicating that blockade of calcineurin signaling to NFAT by GSK-3βS9A was incomplete.

Discussion

Numerous signaling pathways have been shown to be sufficient and necessary to induce cardiac hypertrophy (1, 2, 4, 5). In contrast, relatively little is known of possible antihypertrophic signaling pathways that might be activated therapeutically as a means of inhibiting pathologic forms of cardiac hypertrophy. The results of this study demonstrate that GSK-3β acts as an antihypertrophic kinase that can interfere with hypertrophic signals evoked by diverse hypertrophic stimuli *in vivo*.

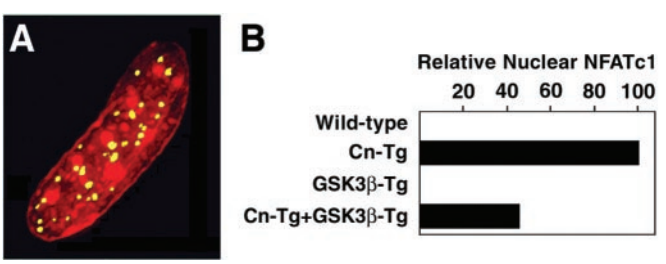

Fig. 4. Calcineurin-induced nuclear localization of NFAT is antagonized by activated GSK-3S9A. (*A*) Representative view of a cardiomyocyte nucleus from an α-MHC-calcineurin transgenic mouse immunostained for NFATc1 expression, as described in *Materials and Methods*. NFAT staining is observed as punctate dots in the nucleus. Propidium iodide (red) was used as a nuclear counter stain. (*B*) The graph shows the relative number of NFAT-positive nuclei in sections of cardiac ventricles of mice of the indicated genotypes. The number of NFAT-positive nuclei in sections from α-MHC-calcineurin transgenic mice was set at 100%.

Potential Mechanisms for the Antihypertrophic Activity of GSK-3. Phosphorylation of the N-terminal regulatory domain of NFAT proteins by GSK-3 and other kinases prevents nuclear import of NFAT and enhances nuclear export (13–15), whereas upon dephosphorylation by calcineurin, NFAT proteins rapidly enter the nucleus (26–29). NFAT nuclear localization is sufficient to induce cardiac hypertrophy (6), suggesting that GSK-3 may diminish the hypertrophic response, at least in part, by preventing nuclear localization of NFAT (Fig. 5). Consistent with this model, localization of NFATc1 to the nucleus in response to activated calcineurin was diminished in the presence of GSK-3βS9A. However, GSK-3βS9A only partially interfered with the ability of calcineurin to drive NFATc1 to the nucleus. Whether the GSK-3-resistant nuclear NFAT accounts for the residual hypertrophy observed in the presence of GSK-3βS9A is unclear.

Although we favor the hypothesis that phosphorylation and resulting cytoplasmic localization of NFAT contributes to the blockade to hypertrophy imposed by activated GSK-3β, GSK-3 can also phosphorylate a variety of other substrates with the potential to influence cardiac growth. For example, GSK-3 can phosphorylate c-Jun, resulting in inhibition of DNA binding (30). Because c-Jun is a target for mitogen-activated protein kinase pathways, which play important roles in hypertrophic signaling (31), its inactivation could interfere with cardiac growth. GSK-3 also phosphorylates c-myc and cyclin D1, resulting in ubiquitin-mediated degradation (32, 33). C-myc is upregulated during cardiac hypertrophy (34) and D-cyclins have been implicated in the transition of cardiomyocytes to hypertrophic growth, through regulation of cyclin-dependent kinases (35). GSK-3 also inhibits glycogen synthesis and protein translation through phosphorylation of glycogen synthase and eukaryotic initiation factor 2B, respectively (16). Any or all of

these targets could contribute to the antihypertrophic effects of GSK-3β.

Several kinases in addition to GSK-3, including protein kinase A, casein kinase-1, MEKK1, and Jun N-terminal kinase (JNK) also phosphorylate the N-terminal regulatory regions of NFAT proteins (13–15, 36). However, cardiac overexpression of protein kinase A induces dilated cardiomyopathy (37) and does not interfere with the effects of calcineurin (C. Antos and E. Olson, unpublished results). Both MEKK1 and JNK have been reported to have both pro- and antihypertrophic activity (38–41). The specificity of the antihypertrophic effects of GSK-3 likely reflects its unique spectrum of targets relative to these other kinases.

GSK-3 is also a key downstream effector in the Wnt signaling pathway (reviewed in refs. 16 and 19). In the absence of Wnt signaling, GSK-3 constitutively phosphorylates β-catenin, a transcriptional cofactor that binds to TCF/LEF transcription factors to up-regulate gene expression. Phosphorylated β-catenin is targeted for degradation by the ubiquitin pathway. Thus, in unstimulated cells, TCF-dependent genes are transcriptionally inactive. Wnt ligands, which act through cell surface Frizzled receptors, initiate an intracellular cascade in which the cytoplasmic protein Dishevelled represses GSK-3 activity, with resulting accumulation of nonphosphorylated β-catenin and activation of TCF-dependent genes. It is currently unknown whether Wnt signaling participates in cardiac hypertrophy.

Cross-Talk and Convergence of Hypertrophic Signaling Pathways. Several protein kinases, including phosphatidyl inositol 3-kinase (PI3-K), Akt/protein kinase B (PKB), p70-S6 kinase, p90-rsk, and protein kinase A have been implicated in hypertrophic signaling in cardiomyocytes (1, 2, 4, 5). All of these kinases can phosphorylate serine-9 of GSK-3 and thereby inhibit kinase activity. This finding raises the possibility that their prohypertrophic actions are mediated, at least in part, by suppressing the antihypertrophic activity of GSK-3.

The ability of GSK-3 to inhibit hypertrophy in response to chronic β-adrenergic stimulation, pressure overload, and calcineurin activation suggests that GSK-3 represents a point of cross-talk and convergence of disparate hypertrophic signaling pathways. Several recent studies have implicated calcineurin as a downstream mediator of hypertrophy in response to β-adrenergic signaling and pressure overload (reviewed in refs. 7–9). Consistent with our results, Marsico *et al.* showed that GSK-3βS9A blunted isoproterenol-induced hypertrophy of primary cardiomyocytes *in vitro* (21).

The regulation of GSK-3 also provides a mechanism for cross-talk between the calcineurin and Akt pathways, because calcineurin activity can stimulate Akt (42), which phosphorylates GSK-3. PI3-K, which also stimulates Akt, is activated in pressure-overload hypertrophy (43). Recently, it was shown that an increase in PI3-K activity can induce cardiac hypertrophy, whereas transgenic overexpression of a dominant-negative PI3-K can decrease cardiac mass (44). In this way, PI3-K signaling can potentiate the calcineurin pathway by relieving the inhibitory influence of GSK-3. Thus, GSK-3 functions as a signal integrator, enabling divergent signaling pathways to cross-talk. This unique function for GSK-3 makes it an attractive target for antihypertrophic drugs.

Uncoupling of Hypertrophic Gene Expression from Cardiomyocyte Hypertrophy. Our results also demonstrate that activated GSK-3β can uncouple the expression of hypertrophic genes from cardiac growth and can regulate different hypertrophic genes in opposing manners. For example, GSK-3βS9A blocked the up-regulation of β-MHC expression that normally accompanies hypertrophy in response to calcineurin activation. In contrast, GSK-3βS9A alone stimulated ANF and BNP expression and enhanced the expression of these genes in response to calcineurin activation. Because ANF and its receptor have been reported to possess potent antihypertrophic activity (45, 46), it is interesting to consider whether its up-regulation by GSK-3βS9A might contribute to the antihypertrophic effects of this kinase. Moreover, recombinant human BNP has been shown to improve decompensated congestive heart failure (47), also suggesting that GSK-3β-mediated up-regulation of natriuretic peptides might be beneficial. The ability of GSK-3βS9A to stimulate ANF expression in the intact heart differs from its repressive effect on ANF expression in primary neonatal cardiomyocytes *in vitro* (20). Of note, cardiac overexpression of a dominant negative form of PI3K in transgenic mice, which would be predicted to enhance GSK-3 activity (see Fig. 5), also up-regulates ANF expression while suppressing cardiac growth (45).

Therapeutic Implications. Our results demonstrate that GSK-3β activity suppresses the cardiac growth response to multiple stimuli. These findings suggest potential approaches for the development of pharmacologic inhibitors of hypertrophy and heart failure targeted at increasing GSK-3 activity. It should also be pointed out that although hypertrophy is often considered to be an adaptive response, cardiac hypertrophy is actually associated with an increased risk for morbidity and mortality (3), indicating that measures to disrupt this cardiac growth response are likely to be clinically beneficial. Indeed, there is evidence that cardiac hypertrophy, at least in the short term, is not required to maintain systolic function in the face of pressure overload, as demonstrated by the preservation of cardiac function in aortic-banded mice treated with calcineurin inhibitors (24) or expressing GSK-3βS9A. Thus, the controlled maintenance of GSK-3 activity may be beneficial in treatment of some forms of cardiomyopathy.

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