Glycogen Synthase Kinase-3β Is a Negative Regulator of Cardiomyocyte Hypertrophy

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Abstract. Hypertrophy is a basic cellular response to a variety of stressors and growth factors, and has been best characterized in myocytes. Pathologic hypertrophy of cardiac myocytes leads to heart failure, a major cause of death and disability in the developed world. Several cytosolic signaling pathways have been identified that transduce prohypertrophic signals, but to date, little work has focused on signaling pathways that might negatively regulate hypertrophy. Herein, we report that glycogen synthase kinase-3β (GSK-3β), a protein kinase previously implicated in processes as diverse as development and tumorigenesis, is inactivated by hypertrophic stimuli via a phosphoinositide 3-kinase–dependent protein kinase that phosphorylates GSK-3β on ser 9. Using adenovirus-mediated gene transfer of GSK-3β containing a ser 9 to alanine mutation, which prevents inactivation by hypertrophic stimuli, we demonstrate that inactivation of GSK-3β is required for cardiomyocytes to undergo hypertrophy. Furthermore, our data suggest that GSK-3β regulates the hypertrophic response, at least in part, by modulating the nuclear/cytoplasmic partitioning of a member of the nuclear factor of activated T cells family of transcription factors. The identification of GSK-3β as a transducer of antihypertrophic signals suggests that novel therapeutic strategies to treat hypertrophic diseases of the heart could be designed that target components of the GSK-3 pathway.

Key words: heart • nuclear factor of activated T cells • adenovirus • endothelin-1 • protein kinase B

Introduction

Many pathologic stimuli induce the heart to undergo adaptive hypertrophic growth that temporarily augments cardiac function. Although the initial hypertrophic response may be beneficial, sustained hypertrophy often undergoes a transition to heart failure, which is a leading cause of mortality and morbidity worldwide, and is characterized by a progressive deterioration in cardiac function. Intensive investigation over the last several years has led to the identification of intracellular signaling pathways that are believed to transduce prohypertrophic signals. However, the nature of the cross-talk between these pathways remains unclear and, more importantly, negative regulators of the hypertrophic response have not been identified.

Glycogen synthase kinase-3 (GSK-3)[1] is a highly conserved protein kinase that is believed to play a critical role in development as a component of the Wnt/wingless pathway, and in a number of human disease states including tumorigenesis, diabetes, and Alzheimer's disease (Parker et al., 1983; Siegfried et al., 1992; Welsh and Proud, 1993; He et al., 1995; Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Unlike most protein kinases, GSK-3 is active in the unstimulated cell and becomes inactivated when cells are stimulated by a variety of mitogens or by the Wnt/wingless pathway (Woodgett, 1994).

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[1]*Abbreviations used in this paper:* ANF, atrial natriuretic factor; ET-1, endothelin-1; GFP, green fluorescent protein; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor-1; ILK, integrin-linked kinase; MOI, multiplicity of infection; NF-AT, nuclear factor of activated T cells; PE, phenylephrine; PI3-K, phosphoinositide 3-kinase; PKB, protein kinase B; SAPK, stress-activated protein kinase.

Many of the targets of GSK-3 that have been identified to date, including c-Jun, cyclin D1, several metabolic enzymes, β-catenin, and at least two nuclear factors of activated T cells (NF-ATs), are repressed by the action of GSK-3, and inactivation of GSK-3 relieves the repression (Boyle et al., 1991; de Groot et al., 1993; Nikolakaki et al., 1993; Aberle et al., 1997; Beals et al., 1997a,b; Diehl et al., 1998; Graef et al., 1999). The list of putative substrates of GSK-3 suggested that this kinase might also play a negative modulatory role in hypertrophy since it negatively regulates the actions of major targets of two cytosolic signaling pathways that have been implicated in the hypertrophic response to pressure overload in the intact animal. These pathways, the calcineurin pathway and a pathway culminating in activation of the stress-activated protein kinases (SAPKs, also known as c-Jun NH_2-terminal kinases or JNKs), activate NF-ATs and c-Jun, respectively (Pulverer et al., 1991; Dérijard et al., 1994; Kyriakis et al., 1994; Beals et al., 1997a).

Calcineurin, activated by calmodulin binding in the presence of elevated cytosolic free [Ca^{2+}], dephosphorylates NF-ATs, exposing the nuclear localization signals (Beals et al., 1997a; Crabtree, 1999; Zhu and McKeon, 1999). NF-ATs then translocate to the nucleus and activate transcription of a number of genes involved in a variety of responses, including the immune response (Rao et al., 1997). When calcium levels return to normal and calcineurin is inactivated, phosphorylation of NF-ATs leads to their rapid export from the nucleus, terminating the signal (Zhu and McKeon, 1999). Although the role of NF-ATs in the hypertrophic response of cardiomyocytes to physiologically relevant stimuli is not clear, Molkentin et al. (1998) were able to induce hypertrophy in transgenic mice by expressing activated NF-ATc4. These data suggest that calcineurin's prohypertrophic effects are mediated, at least in part, via activation of one or more NF-ATs. More recently, calcineurin-induced activation of NF-ATc1 (NF-AT2/c) was shown to play a role in skeletal myocyte hypertrophy (Musaro et al., 1999).

GSK-3β has been reported to regulate nuclear/cytoplasmic partitioning of various NF-ATs. GSK-3β has been shown to induce nuclear export of transfected NF-ATc1 in COS cells, and of transfected NF-ATc4 (NF-AT3) in hippocampal neurons (Beals et al., 1997b; Graef et al., 1999). Although several other kinases also have been implicated in regulation of NF-AT subcellular localization (Zhu et al., 1998; Chow et al., 2000; Porter et al., 2000), these data raise the possibility that GSK-3β could exert an antihypertrophic effect in the heart by affecting nuclear/cytoplasmic partitioning of endogenous NF-ATs in cardiac myocytes.

c-Jun, a major SAPK target (Pulverer et al., 1991; Kyriakis et al., 1994), is also negatively regulated by phosphorylation by GSK-3 (Boyle et al., 1991; de Groot et al., 1993; Nikolakaki et al., 1993). Phosphorylation reduces the DNA binding activity of c-Jun, and thus the activity of AP-1 (a heterodimer of c-Jun and c-Fos family members). Since the SAPKs recently have been shown to be necessary for the hypertrophic response of neonatal cardiomyocytes to endothelin-1 (ET-1) and for the development of pressure overload-induced hypertrophy in the intact rat (Choukroun et al., 1998, 1999), inhibition of activity of one of the primary targets of the SAPKs, AP-1, could be another mechanism whereby GSK-3 might negatively regulate hypertrophy.

Herein, we explore the role of GSK-3β in the hypertrophic response of cardiomyocytes. Our data indicate that inhibition of GSK-3β is a critical step in the development of a cardiac hypertrophic response.

Materials and Methods

Materials

Antibodies used were: anti-GSK-3β mAb (Transduction Laboratories), antiatrial natriuretic factor (anti-ANF; Peninsula Laboratories), anti-α-actinin mAb (Sigma-Aldrich), rabbit anti-NF-ATc1 (K-18), which recognizes all NF-AT family members (Santa Cruz Biotechnology), antiphospho Ser 9 GSK-3β that specifically recognizes Ser 9 phosphorylated GSK-3β (New England Biolabs), and Cy3-conjugated anti-rabbit and anti-mouse antibodies (BioRad). Other reagents included glycogen synthase peptide-2 (Upstate Biotechnology, Inc.), ET-1 and phenylephrine (PE; Sigma-Aldrich), and insulin-like growth factor-1 (IGF-I; Calbiochem).

Cell Culture

Spontaneously beating neonatal myocytes were prepared from 1–2-d-old rats and cultured in F-10 medium in the presence of 5% FBS and 10% horse serum as previously described (Choukroun et al., 1998).

Adenoviral Vectors

Construction of Recombinant Adenoviral Vector Carrying the GSK-3βA9 cDNA. The cDNA encoding GSK-3βA9, carrying a Ser-to-Ala substitution at Ser 9 in the NH_2-terminal region of GSK-3β, and an HA epitope tag at the COOH terminus was created by PCR as described (Stambolic and Woodgett, 1994). The cDNA was subcloned into the pAdTRACK-CMV shuttle vector (obtained from B. Vogelstein, Johns Hopkins University, Baltimore, MD) that encodes green fluorescent protein (GFP) from one CMV promoter and the gene of interest from a second CMV promoter (He et al., 1998). AdGSK-3βA9, the recombinant adenovirus, was prepared using the AdEASY system as described (He et al., 1998). The recombinant virus was propagated in 293 cells and high titer stocks ($\geq 10^{12}$ particles/ml) were purified by CsCl density gradient centrifugation.

Other Adenoviral Vectors. AdβgalEGFP (herein referred to as AdGFP), carrying the *Escherichia coli* LacZ gene in addition to the GFP gene, was used as a control virus. AdBD110, which encodes the 110-kD catalytic subunit of phosphoinositide 3-kinase (PI3-K), rendered constitutively active by including in-frame the p110-binding domain of human p85 (amino acids 474–552), has been previously described in detail (Matsui et al., 1999). When cardiomyocytes are infected with AdBD110, they have constitutively elevated levels of 3-phosphorylated phosphoinositides and increased activity of PKB/Akt (Matsui et al., 1999). AdPKB/Akt, encoding protein kinase B (PKB)/Akt made constitutively active by the addition of a myristylation signal at the NH_2 terminus of the kinase, was kindly provided by Dr. Thomas Franke (Columbia University, New York, NY) and has been described in detail (Matsui et al., 1999).

Cell Fractionation

Cells were fractionated by hypotonic lysis. In brief, cells were suspended in lysis buffer containing Hepes (20 mM, pH 7.5) and NaCl (10 mM) with phosphatase and protease inhibitors. After 15 min on ice, lysates were spun at 2,500 rpm for 5 min in an Eppendorf centrifuge. The pellet (nuclear fraction) was washed twice in lysis buffer, and then the supernatant and pellet were spun at 14,000 rpm for 10 min. Protein concentrations of the cytosolic and nuclear fractions were equalized, and then SDS sample buffer was added to a final concentration of 1×.

Immunoblot Analysis

For Western blot analysis, cell lysates were matched for protein concentration and were then separated by SDS-PAGE and transfered to Hybond-C extra (Amersham Pharmacia Biotech). The membranes were blocked in 5% nonfat milk and then incubated with the indicated antibodies for 1 h at room temperature. Antibody binding was detected with a peroxidase-conjugated goat anti–rabbit or anti–mouse IgG and chemiluminescence.

Immune Complex Kinase Assay of GSK-3β

For the studies of GSK-3β activity in aortic banded hearts, the left ventricle was pulverized under liquid nitrogen, homogenized with a polytron in lysis buffer containing protease and phosphatase inhibitors (Pombo et al., 1994; Choukroun et al., 1999), and then briefly sonicated. After 15 min on ice with vortexing, the samples were centrifuged at 100,000 *g* for 1 h at 4°C. Supernatants from heart lysates, or from lysates of neonatal cardiomyocytes in culture, were matched for protein concentration, and were incubated with anti-GSK-3β mAb or anti-HA mAb for 2 h, and then complexes were collected with protein G–Sepharose beads for an additional 1 h. Beads were washed six times in lysis buffer and three times in assay buffer, and then were incubated for 20 min at 30°C with glycogen synthase peptide-2 (50 μM) and 100 μM γ[^{32}P]ATP (3,000–4,000 cpm/pmol) in the presence of 10 mM $MgCl_2$. Contents of the assays were spotted onto P81 phosphocellulose papers that were washed and then subjected to liquid scintillation counting. Kinase activity was reduced to background levels when 10 mM LiCl was included in the reaction mix, suggesting the activity measured was GSK-3β and not a contaminating kinase.

[^{3}H]-leucine Incorporation

Neonatal cardiomyocytes were infected with AdGSK-3βA9 or AdGFP in F-10 medium containing 0.1% FCS. 36 h later, cells in triplicate wells of 12-well plates were stimulated with ET-1 (100 nM) for 36 h in serum-free F-10 medium and then incubated in the same medium with 1.0 μCi/ml [^{3}H]-leucine for an additional 12 h. The cells were processed as described (Choukroun et al., 1998), and [^{3}H]-leucine incorporation was determined by liquid scintillation counting.

Immunocytochemistry

Cardiomyocytes, grown on laminin-coated plastic coverslips, were infected with either AdGFP or AdGSK-3βA9. 36 h later, they were exposed to ET-1 or PE in serum free medium. For assessment of sarcomere organization and ANF expression, the cells were fixed 48 h later for 10 min with 4% parformaldehyde/5% sucrose in PBS. Coverslips were processed as described (Molkentin et al., 1998). For sarcomere staining, coverslips were incubated in a 1:400 dilution of anti–α-actinin mAb and for ANF staining in a 1:400 dilution of anti-ANF antibody in blocking solution. Coverslips were then incubated in a Cy3-conjugated secondary antibody diluted 1:800 in blocking solution for 1 h at room temperature. Cells were photographed using a Nikon FXA photomicroscope. Figures were prepared using Canvas 6.0.1 (Deneba Systems, Inc.) and were then transferred to Adobe Photoshop 5.5 for printing.

Statistical Analysis

Data are expressed and presented in the figures as mean ± SEM. A *t* test was used to compare the means of normally distributed continuous variables. A value of $P < 0.05$ was chosen as the limit of statistical significance.

Results

GSK-3β Is Inhibited by Hypertrophic Stimuli

If GSK-3β plays an important role in the hypertrophic response, its activity should be inhibited by hypertrophic stimuli. Therefore, we exposed neonatal rat cardiomyocytes to ET-1 or, as a positive control, IGF-1, another hypertrophic agent known to inhibit GSK-3β. Cell lysates were prepared and subjected to immunoprecipitation with an anti-GSK-3β antibody, followed by assay with the glycogen synthase-2 peptide as substrate. IGF-1 produced a 45% decrease in GSK-3β activity, consistent with prior observations using insulin in various cell lines (Cross et al., 1995; Moule et al., 1997). In response to ET-1 (100 nM), GSK-3β was inhibited by as much as 60% at 40 min. Thereafter, GSK-3β activity returned toward control levels, but some inhibition persisted for at least 90 min (Fig. 1 A). Inhibition with PE (20–30%) was not as marked as

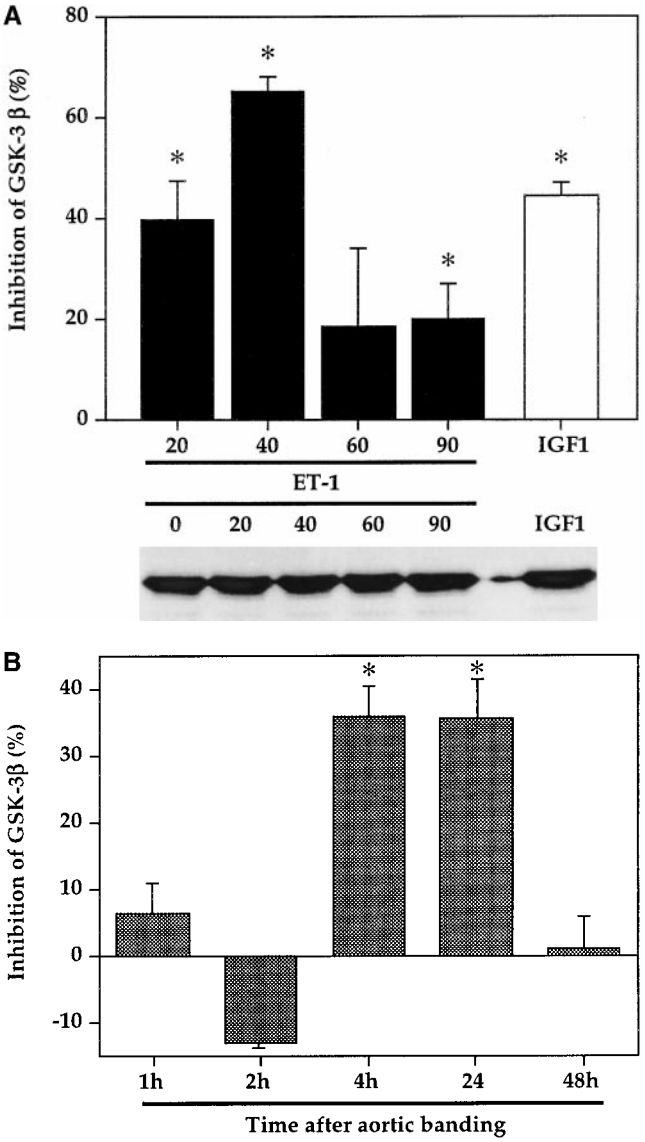

Figure 1. Inhibition of GSK-3β by ET-1 in neonatal cardiomyocytes in culture (A), and by pressure overload in vivo (B). A, Cardiomyocytes were exposed to IGF-1 (40 ng/ml) for 20 min, or to vehicle or ET-1 (100 nM) for 20, 40, 60, or 90 min, followed by immune complex kinase assays for GSK-3β activity. Percent inhibition of GSK-3β activity is expressed relative to vehicle controls. Western blot of total GSK-3β in the lysates is shown below the graph. $n = 3$ experiments, assayed in duplicate. $^*P < 0.01$ vs. vehicle control. B, Rats were subjected to aortic banding or sham banding, and then were killed at 2, 4, 24, or 48 h after banding. GSK-3β immune complex kinase assays were performedon myocardial lysates, and percent inhibition of kinase activity is expressed relative to sham banded animals. $n = 3$ animals per condition. $^*P < 0.01$ vs. sham banded animals.

with ET-1, but was significant (data not shown). Although PE-induced inhibition is less than the inhibition seen with IGF-1, this percent inhibition is equivalent to that seen with NGF and HGF (20–30% inhibition), and this degree of inhibition is believed to play an important role in the antiapoptotic effect of NGF in PC12 cells (Pap and Cooper, 1998) and in the HGF-induced accumulation of β-catenin in mouse mammary epithelial cells (Papkoff and

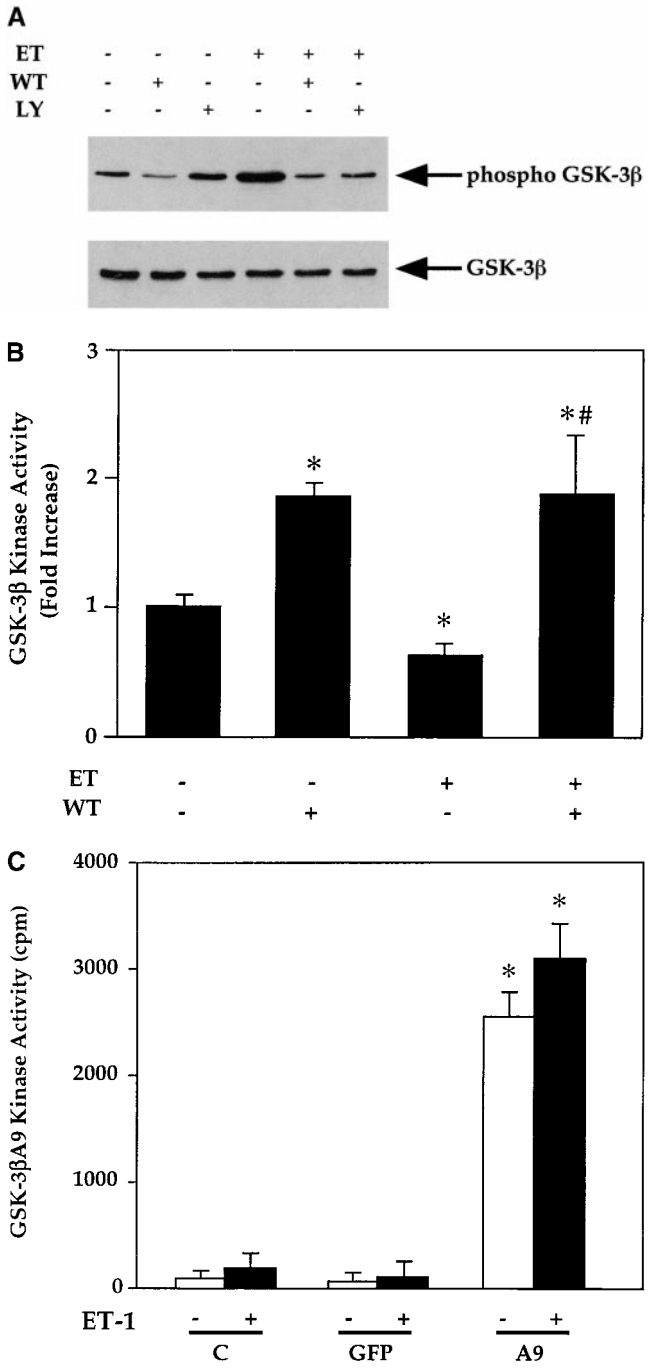

Figure 2. Hypertrophic stimuli inhibit GSK-3β via phosphorylation of Ser 9 by a PI3-K-dependent protein kinase. A, ET-1 induces Ser 9 phosphorylation of GSK-3β by a PI3-K–dependent kinase. Cells were pretreated for 30 min with vehicle (DMSO), wortmannin (100 nM), or LY294002 (10 μM), and then were stimulated with vehicle (ET−) or ET-1 (ET+) for 40 min. Western blotting of whole cell lysates was performed with antiphospho Ser 9 GSK-3β and, to confirm that equivalent amounts of protein were loaded, with anti-GSK-3β. Experiment shown is representative of three. B, ET-1–induced inactivation of GSK-3β is PI3-K-dependent. Cardiomyocytes were pretreated for 30 min with wortmannin (100 nM) or vehicle (DMSO), and then were stimulated with ET-1 or vehicle for 40 min, followed by GSK-3β immune complex kinase assay. $n = 3$ experiments, assayed in duplicate. $*P < 0.01$ vs. control (ET−/WT−). $\#P < 0.01$ vs. ET-1 alone (ET+/WT−). C, GSK-3βA9 is not inhibited by ET-1 in cardiomyocytes. Cells were transduced with AdGSK-3βA9 (A9) or AdGFP (GFP) at an MOI of 100 pfu/cell, or no virus (C). 36 hours later, cells were stimulated with vehicle or ET-1 for 30 min, lysates were prepared and anti-HA immune complex kinase assays were performed. Experiments were performed in parallel with the experiments presented in Fig. 1 A, demonstrating significant inhibition of GSK-3β by ET-1. $*P < 0.01$ vs. respective no virus control (C) and GFP virus control.

Aikawa, 1998). These data suggest that the degree of inhibition of GSK-3β by ET-1 and PE in cardiomyocytes is sufficient to have potentially important biological effects.

To determine whether GSK-3β might play a role in the hypertrophic response to a physiologically relevant stimulus in vivo, rats were subjected to aortic banding or sham banding as described (Choukroun et al., 1999), and GSK-3β immune complex kinase assays were performed on myocardial lysates at various times after banding. We found that GSK-3β activity was significantly reduced in response to pressure overload and inhibition persisted for 24 h (Fig. 1 B). Taken together, these data are consistent with a possible role for GSK-3β in the hypertrophic response of cardiomyocytes in culture and in pressure overload-induced hypertrophy in vivo.

Mechanism of Inhibition of GSK-3β by Hypertrophic Stimuli

Several mechanisms of inhibition of GSK-3 have been described. Insulin and IGF-1 inactivate GSK-3 via phosphorylation of a serine residue in the NH_2-terminal region of the kinase (Ser 9 for GSK-3β and Ser 21 for GSK-3α; Stambolic and Woodgett, 1994). This is mediated by a PI3-K-dependent kinase, possibly either PKB/Akt or the integrin-linked kinase (ILK; Cross et al., 1995; Delcommenne et al., 1998). Other mechanisms, including one mediated by Ca^{2+} and a Ca^{2+}/calmodulin-dependent protein kinase kinase (Yano et al., 1998), and an ill-defined mechanism employed by the Wnt/wingless pathway, possibly involving protein kinase C (Cook et al., 1996), also inactivate GSK-3, but these pathways are not PI3-K-dependent and do not result in phosphorylation of Ser 9. Therefore, we determined the mechanism of inhibition of GSK-3β by hypertrophic stimuli. We found that ET-1 induced pronounced phosphorylation of GSK-3β on Ser 9, and that this phosphorylation was blocked by the PI3-K inhibitors, wortmannin or LY294002 (Fig. 2 A). The effect of the PI3-K inhibition on Ser 9 phosphorylation exactly correlated with the effect on GSK-3β kinase activity, since wortmannin prevented the ET-1–induced inactivation of GSK-3β (Fig. 2 B). These data strongly suggest that the ET-1–induced inhibition of GSK-3β is mediated via phosphorylation of Ser 9 by a PI3-K-dependent kinase.

To confirm that phosphorylation of Ser 9 was the mechanism of inactivation of GSK-3β, we created an adenovirus encoding GSK-3β with a Ser 9 to Ala mutation that renders the kinase resistant to inhibition by Ser 9 kinases, and then determined whether GSK-3βA9 was inhibited in response to ET-1. Cells were transduced with AdGSK-3βA9, AdGFP (as a control), or no virus. 36 h later, cells were exposed to ET-1 for 40 min, followed by immunoprecipitation

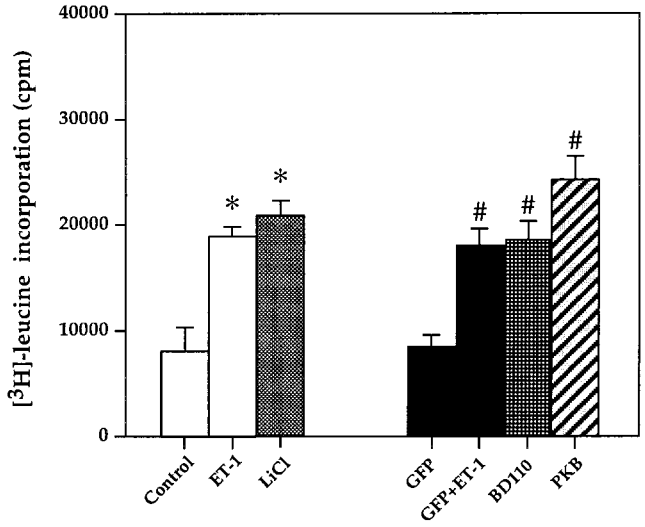

Figure 3. Gene transfer of constitutively active PI3-K (BD110) or PKB/Akt, or treatment with LiCl induces protein synthesis in neonatal rat cardiomyocytes. Neonatal cardiomyocytes were treated with vehicle (Control), ET-1, or LiCl for 48 h (left side), or were transduced with AdBD110 or AdPKB/Akt, or with control virus (AdGFP) for 48 h. Leucine incorporation was determined over the last 12 h of the incubation. $*P < 0.01$ vs. vehicle control. $\#P < 0.01$ vs. virus control (GFP).

with anti-HA mAb and immune complex kinase assay. In contrast to endogenous GSK-3β (Figs. 1 A and 2 B), GSK-3βA9 was not inhibited by ET-1 (Fig. 2 C). These data confirmed a critical role for Ser 9 phosphorylation in the ET-1–induced inactivation of GSK-3β. In addition, they demonstrated that we could employ the virus to study the role of GSK-3 inhibition in the hypertrophic response to ET-1.

Inhibitors of GSK-3 Induce Hypertrophic Responses in Cardiac Myocytes

If the ET-1–induced inhibition of GSK-3 were mediated by activation of the PI3-K/PKB pathway, and if this inhibition were important in the hypertrophic response, then directly activating the PI3-K/PKB pathway should induce hypertrophic responses. To initially explore this question, we induced inhibition of GSK-3 by adenovirus-mediated gene transfer of either the constitutively active mutant of PKB/Akt (AdPKB/Akt), or the constitutively active PI3-K (AdBD110), which produces persistent activation of endogenous PKB/Akt (Matsui et al., 1999), and determined their effects on protein accumulation in neonatal myocytes. Adenoviral gene transfer of either BD110 or activated PKB/Akt significantly increased protein accumulation, demonstrating that activation of the PI3-kinase/Akt pathway is sufficient to induce hypertrophic responses (Fig. 3). In addition, we employed LiCl, which has been used to directly inhibit GSK-3 in many contexts (see below). Even in the absence of a hypertrophic stimulus, LiCl was sufficient to induce protein accumulation in cardiac myocytes (Fig. 3). Whereas BD110, PKB/Akt, and LiCl have effects in cells in addition to inhibiting GSK-3, the data are consistent with a possible role for the PI3-K/Akt/GSK-3 pathway in the hypertrophic response.

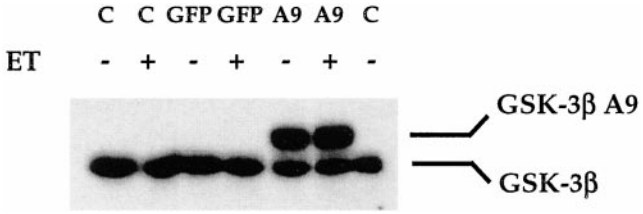

Figure 4. Expression of GSK-3βA9 in neonatal cardiomyocytes. Neonatal myocytes were transduced with AdGFP (GFP) or AdGSK-3βA9 (A9) at an MOI of 100. 36 h later, cell lysates were prepared, and Western blotting with anti-GSK-3β mAb was performed. Due to the HA tag, GSK-3βA9 runs at a slightly higher Mr than does the endogenous kinase. Of note, LiCl did not affect expression of GSK-3βA9 (data not shown).

Expression of GSK-3βA9 Blocks the Hypertrophic Response to ET-1 and PE

Expression of GSK-3βA9. To address our primary question, whether inactivation of GSK-3β is necessary for the hypertrophic response to physiologically relevant stimuli, we expressed GSK-3βA9 in neonatal cardiomyocytes via adenoviral gene transfer and determined its effect on the hypertrophic response. GSK-3βA9 was readily expressed in culture using multiplicities of infection (MOIs) of 50–125 pfu/cell. At an MOI of 100 pfu/cell, expression levels of GSK-3βA9 were only slightly greater than levels of endogenous GSK-3β (Fig. 4), and transduction efficiency was ~85% (not shown). It is important to note that the expression levels that we achieved at an MOI of 100 pfu/cell did not produce marked elevations in total cellular GSK-3β activity. When we measured total GSK-3β activity in cells infected with AdGSK-3βA9, activity was increased only 1.8 ± 0.2-fold over cells infected with control virus (AdGFP). This level of activity is in distinct contrast to activity levels seen after gene transfer or transfection of constitutively active kinases that are normally off in the resting cell. In these cells, total kinase activity is often many fold greater than endogenous activity.

Effect of GSK-3βA9 on Hypertrophic Responses. If inhibition of GSK-3β is important in the hypertrophic response of cardiomyocytes, preventing inactivation of GSK-3 should block the hypertrophic response. Therefore, we determined whether gene transfer of GSK-3βA9 blocked the hypertrophic response to ET-1 and PE. For these studies, we took advantage of the fact that we made AdGSK-3βA9 with the pAdTRACK/pAdEASY system (He et al., 1998), which allows expression of GFP from a separate promoter in the virus. We identified transduced cells by their green fluorescence, and could then compare hypertrophic responses in cells that were successfully transduced with those that were not transduced. To summarize the data presented below, we found that expression of GSK-3βA9 significantly inhibited ET-1– and PE-induced hypertrophy and, importantly, that LiCl, which inhibits endogenous GSK-3 and GSK-3βA9, reversed the effects of gene transfer of GSK-3βA9.

Enhanced organization of sarcomeres, which characterizes the hypertrophic response of neonatal cardiomyocytes, was markedly inhibited by expression of GSK-3βA9 (Fig. 5, a and b). However, in myocytes expressing GSK-3βA9 that were also treated with LiCl (10 mM), ET-1– and

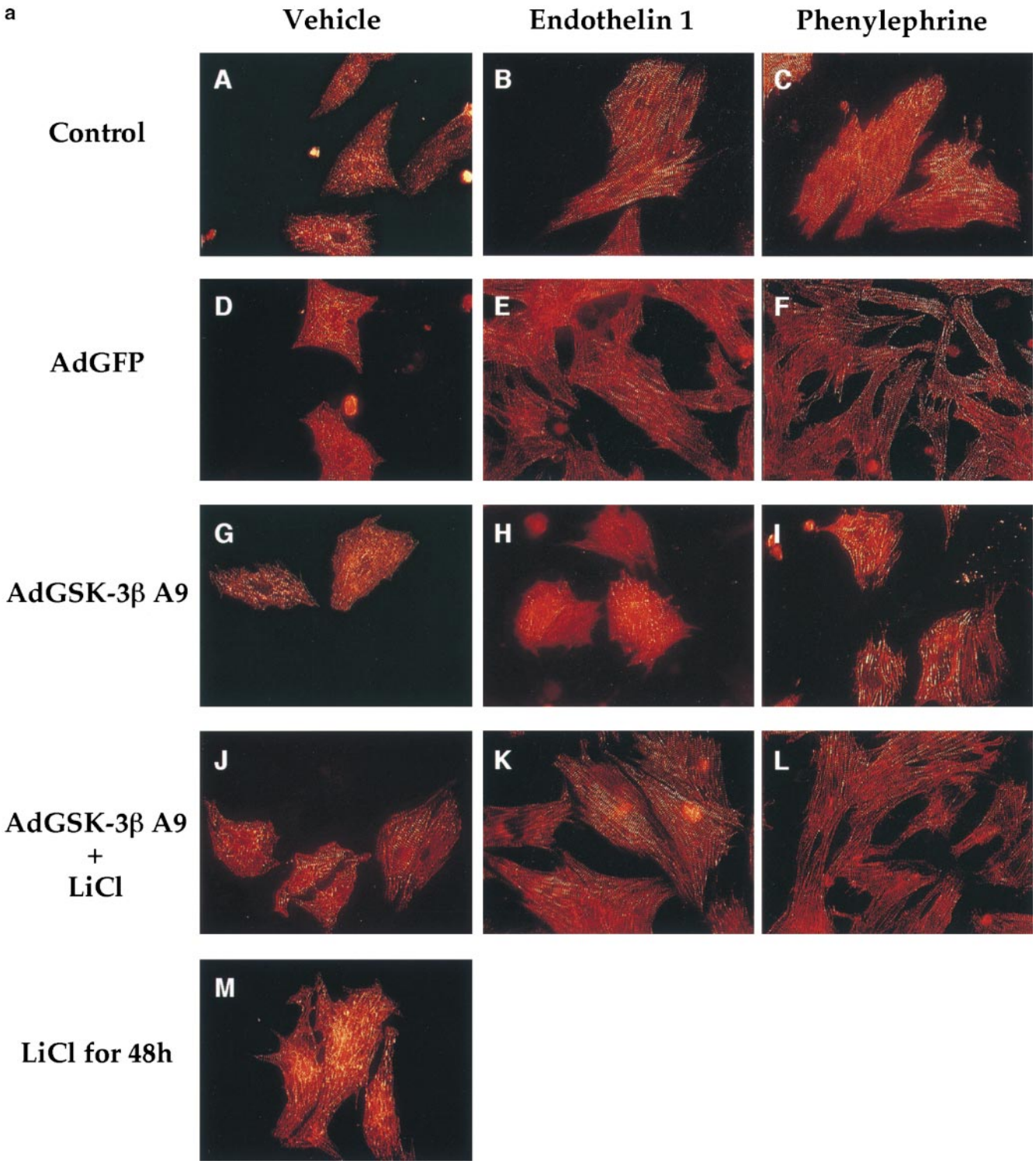

Figure 5. Expression of GSK-3βA9 inhibits ET-1– and PE-induced sarcomere organization. a, Cells were transduced with AdGFP or AdGSK-3βA9, or no virus (Control). 36 h later, cells were treated with vehicle, ET-1, or PE, with or without LiCl present. Sarcomere organization was determined 48 h later. Expression of GSK-3βA9, as opposed to expression of GFP, markedly reduces ET-1– and PE-induced sarcomere organization (compare H with E, and I with F), and this effect of GSK-3βA9 is reversed by LiCl (10 mM; compare K with H, and L with I). M shows the moderate sarcomere organization that is induced by 48 h of LiCl in the absence of ET-1. b, Quantitation of sarcomere organization. Cardiomyocytes, which had been transduced with AdGFP or AdGSK-3βA9 (A9), were treated with vehicle (C), ET-1, or PE, in the presence or absence of LiCl (10 mM) (A9/Li). 48 h later, myocytes ($n \geq 100$ per experiment, $n = 3$ experiments) were scored for the presence or absence of highly organized sarcomeres. $*P < 0.01$ vs. control/GFP. $\#P < 0.01$ vs. respective GFP and A9/Li conditions.

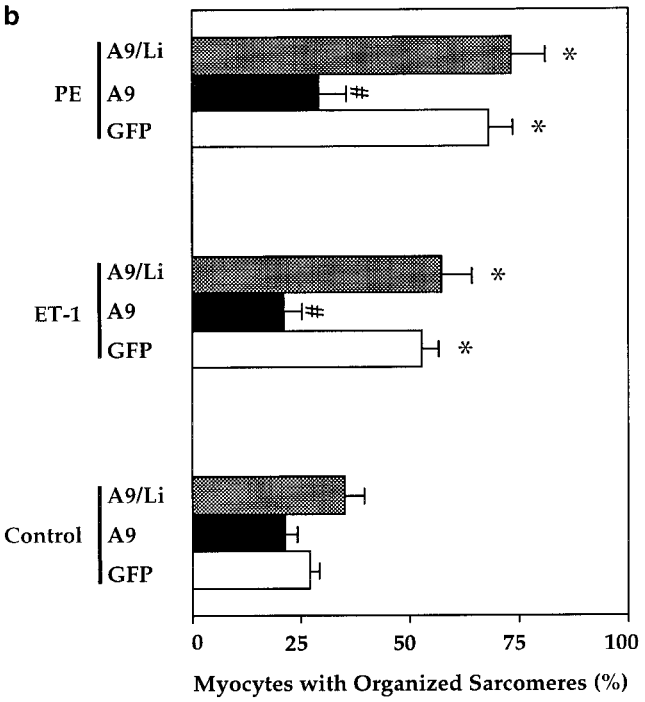

Figure 5 (continued).

PE-induced sarcomere organization was restored (Fig. 5, a and b). Of note, treatment of myocytes with LiCl alone, in the absence of ET-1 or PE, induced only moderate sarcomere organization (Fig. 5 a), suggesting that inhibition of GSK-3β is necessary, but is not sufficient for the full expression of this relatively complex component of the hypertrophic response that requires the coordinate expression of a number of genes.

Next we examined the role of the GSK-3β pathway in the induction of ANF, one of the marker genes that is upregulated in response to most hypertrophic stimuli. We found that ET-1 induced expression of ANF (Fig. 6 a), but that gene transfer of GSK-3βA9 markedly inhibited the ET-1–induced expression of ANF (Fig. 6, b, c, and d). Again, LiCl completely reversed the effects of expression of GSK-3βA9. Although LiCl alone was not sufficient to induce full sarcomere organization (Fig. 5 a), LiCl was sufficient, even in the presence of GSK-3βA9, to induce ANF expression, suggesting that inhibition of GSK-3β is not only necessary, but may also be sufficient for this component of the response (Fig. 6, b, c, and d).

To determine whether inhibition of GSK-3β is necessary for the enhanced protein accumulation that is characteristic of the hypertrophic response, we measured ET-1–induced [^{3}H]-leucine incorporation in cells transduced with AdGSK-3βA9. Expression of GSK-3βA9 significantly reduced ET-1–induced [^{3}H]-leucine incorporation (Fig. 7).

Mechanisms of Action of GSK-3β

The hypertrophic response is an enormously complex response that is regulated by multiple transcription factors acting on multiple genes. This complexity makes it difficult to identify critical roles for individual transcription factors. However, several lines of evidence, in addition to the findings described above with the transgenic mouse expressing an activated mutant of NF-ATc4, suggest NF-ATs play a role in the response. In neonatal rat cardiomyocytes in culture, cyclosporin A blocks ANF induction by angiotensin II and PE (Molkentin et al., 1998), and by ET-1 (data not shown). These data confirm that calcineurin is critical to this component of the response and are compatible with a role for one or more NF-ATs in ANF induction. Furthermore, adenovirus-mediated gene transfer of activated NF-ATc4 (NF-ATc4Δ317; Molkentin et al., 1998), in the absence of hypertrophic stimuli, induces ANF expression and sarcomere organization (data not shown).

We found that ET-1 induced marked nuclear translocation of an endogenous NF-AT of molecular mass ~95 kD (Fig. 8 A). Specific antibodies to the various NF-ATs are not adequate for use in the rat. However, of the three NF-ATs expressed in the heart, NF-ATc1 (NF-ATc), NF-ATc3 (NF-AT4/x), and NF-ATc4 (NF-AT3; J. Molkentin, manuscript in preparation), this molecular mass is most compatible with NF-ATc1. NF-AT first appeared in the nucleus at ~30 min after ET-1 (Fig. 8 A), corresponding to the time of maximal inhibition of GSK-3β activity (Fig. 1 A). Nuclear NF-AT levels declined after 120 min, a time when GSK-3β activity was returning toward normal (Fig. 8 A). Thus, the time courses of endogenous NF-AT nuclear localization and GSK-3β activity are compatible with a role for GSK-3β in regulating nuclear/cytoplasmic partitioning of NF-AT.

Prior studies examining the role of GSK-3β in the nuclear export of NF-ATs have employed overexpression of GSK-3β. In these studies, GSK-3β is found in the nucleus and induces export of NF-ATs (Beals et al., 1997b; Graef et al., 1999). However, it is not clear whether endogenous GSK-3β also translocates to the nucleus in a stimulus-dependent manner. We found that in the unstimulated cardiomyocyte, little GSK-3β is nuclear localized (Fig. 8 B). ET-1 induced a pronounced translocation of GSK-3β to the nucleus (Fig. 8 B). This translocation was evident as early as 30 min and persisted until after 90 min. These data confirm that endogenous GSK-3β translocates to the nucleus in response to hypertrophic stimuli, colocalizing with its putative target, NF-AT.

Thus, the time courses of GSK-3β activity and nuclear localization, and the time course of NF-AT nuclear localization, were consistent with a role for GSK-3β in regulating NF-AT nuclear/cytoplasmic partitioning, following hypertrophic stimuli. To directly address whether GSK-3β modulated NF-AT activity in response to hypertrophic stimuli, we examined the effects of expressing GSK-3βA9 on the nuclear/cytoplasmic partitioning of NF-AT in cardiomyocytes exposed to ET-1. As noted above, NF-AT first appeared in the nucleus at ~30 min after ET-1 in control (Fig. 8 A) and AdGFP-infected cells (Fig. 8 C). Expression of GSK-3βA9 significantly delayed the appearance of NF-AT in the nucleus, compatible with retardation of entry by cytosolic GSK-3β (Fig. 8 C). By 60 min, however, the amount of intranuclear NF-AT was equivalent in cells infected with AdGSK-3βA9 and AdGFP (Fig. 8 C), suggesting the inhibitory effect of GSK-3βA9 was overcome by activated calcineurin. A significant fraction of the NF-AT remained intranuclear at 120 min after ET-1 in control (Fig. 8 A) and AdGFP-infected cells (Fig. 8 C), but expression of GSK-3βA9 accelerated its export from the nucleus such that little remained intranuclear at 120 min (Fig.

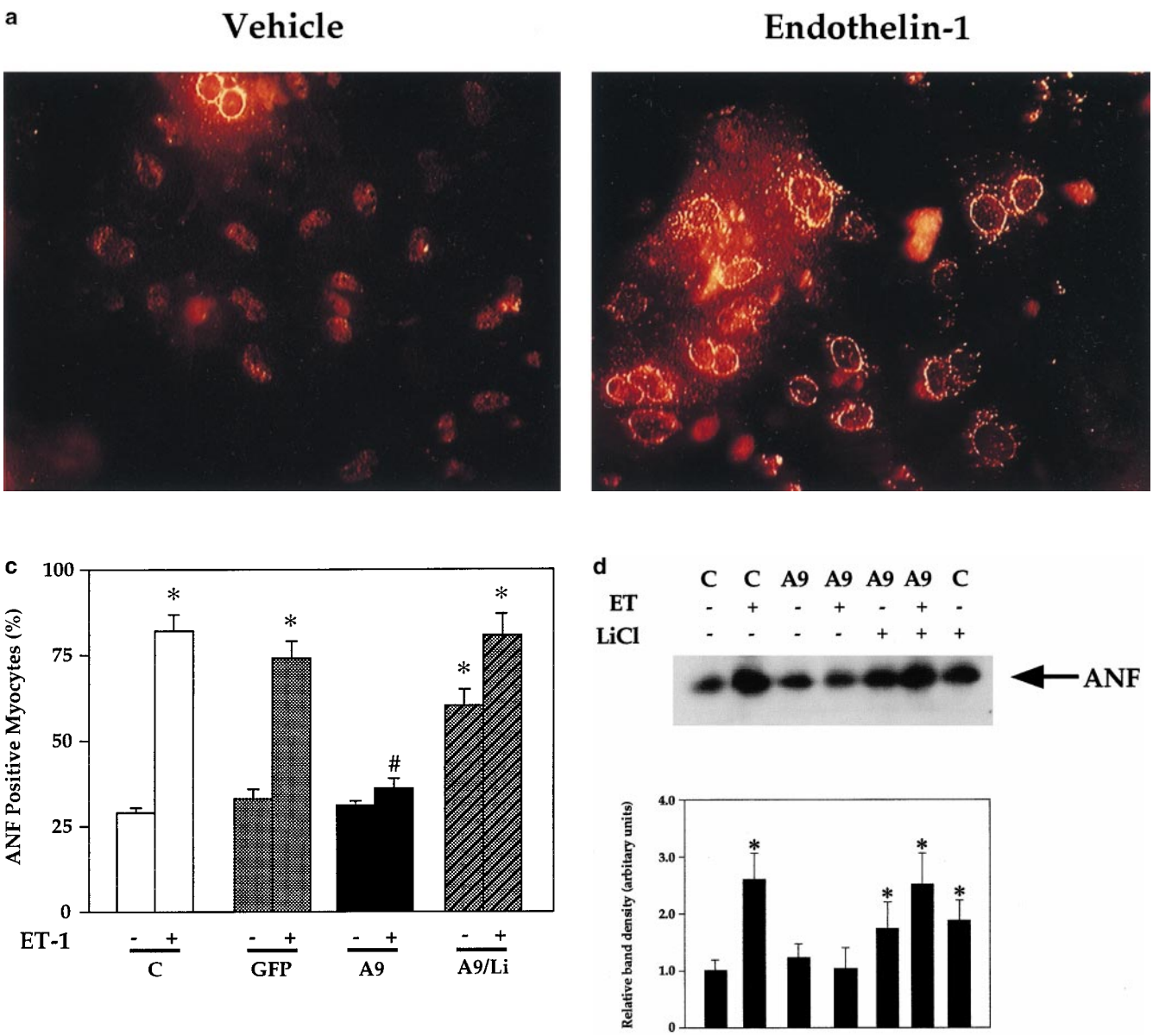

Figure 6. Expression of GSK-3βA9 inhibits ET-1–induced ANF expression. A, ET-1 induces ANF expression in cardiomyocytes. Myocytes were treated with vehicle or ET-1, and 48 h later were stained for ANF expression. Two vehicle-treated cells were positive for ANF expression whereas the majority of ET-1–treated cells were positive. B, Effect of GSK-3βA9 on ANF expression. Cardiomyocytes were transduced with either AdGFP or AdGSK-3βA9 (MOI = 50 pfu/cell), and then were exposed to ET-1 with or without LiCl present. The left panels show staining for ANF expression and the right panels show GFP expression, thus identifying the cells in each field that were successfully transduced with the virus. The control virus (AdGFP) does not prevent ET-1–induced ANF expression (A and B). For the cells transduced with AdGSK-3βA9, in the absence of LiCl (C and D), of the seven cells expressing ANF, none were transduced with AdGSK-3βA9 (compare C and D). In contrast, none of the cells that were transduced with AdGSK-3βA9 (green) express ANF. However, numerous cells transduced with AdGSK-3βA9 express ANF when LiCl is present (E and F), and LiCl restores ET-1 responsiveness in GSK-3βA9-expressing cells (G and H). C, Quantitation of the effect of GSK-3βA9 on ANF expression. Cells were transduced with AdGFP (GFP), or AdGSK-3βA9 (A9), and then were exposed to ET-1 or vehicle, with or without LiCl present (A9/Li). The percent of transduced cells (i.e., GFP positive) expressing ANF was then determined for the various conditions. Also shown is ANF expression in control cells not transduced with virus (C). At least 100 myocytes were scored for each experiment. $*P < 0.01$ vs. vehicle-treated control cells (C/ET−), GFP infected cells (GFP/ET−), and A9 infected cells (A9/ET−). $\#P < 0.01$ vs. all ET-1–treated cells. D, Immunoblot confirming the findings from B and C. Cardiomyocytes were infected with AdGSK-3βA9 (A9) (MOI = 100 pfu/cell) or no virus (C), and then were exposed to vehicle or ET-1, with or without LiCl present. Expression of GSK-3βA9 prevented ET-1–induced ANF expression, and LiCl reversed the inhibition. Also shown is the effect of LiCl alone, which induces moderate ANF expression. Below the immunoblot is a graph showing quantitation of ANF expression by band densitometry from $n = 3$ experiments. $*P < 0.05$ vs. C, A9/Control, and A9/ET.

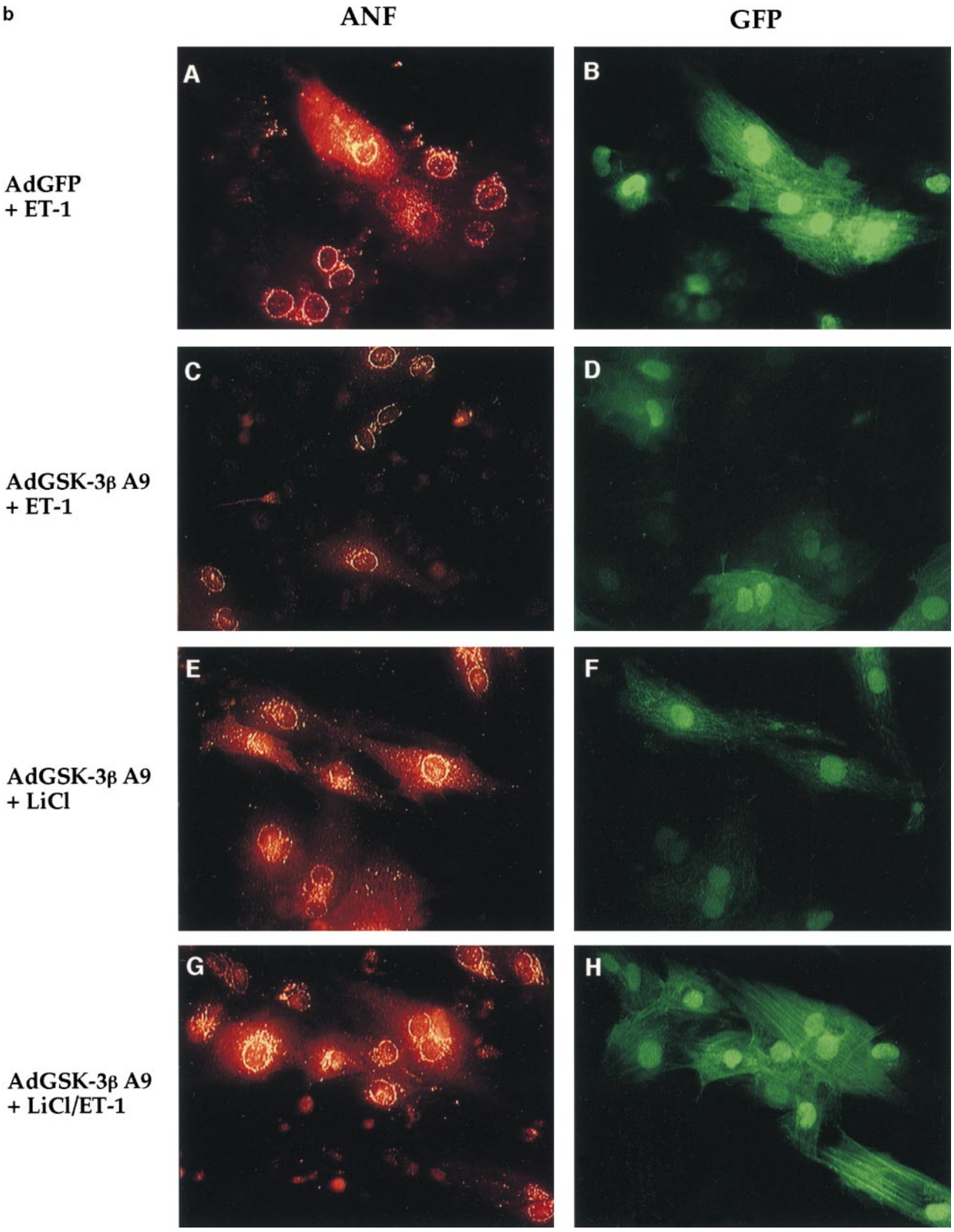

Figure 6 (continued).

8 C). Thus, expression of GSK-3βA9 significantly reduced the duration of NF-AT nuclear localization, both by retarding entry into and enhancing exit from the nucleus. These data suggest GSK-3β modulates the hypertrophic response of cardiac myocytes in part by regulating the nuclear/cytoplasmic partitioning of NF-AT.

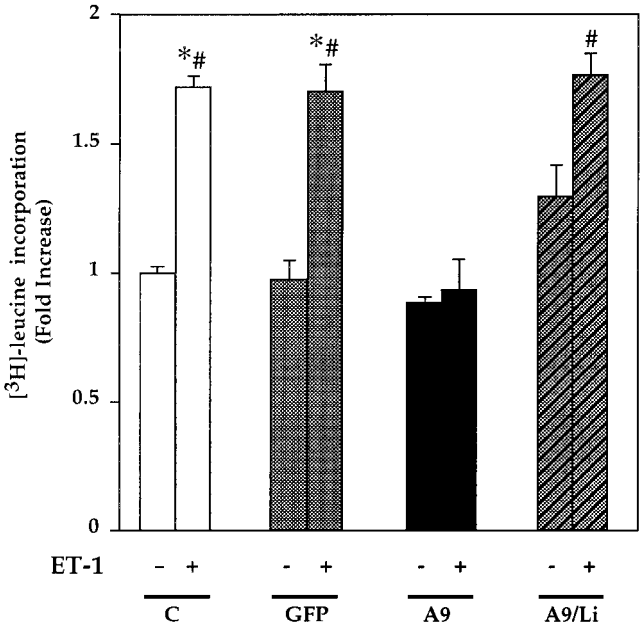

Figure 7. Effect of GSK-3βA9 on ET-1–induced protein synthesis. Neonatal cardiomyocytes were transduced at an MOI of 125 pfu/cell with AdGSK-3βA9 or AdGFP for 36 h, or no virus (C), and then were treated with ET-1 (+) or vehicle (−), with or without LiCl present. [^{3}H]-leucine incorporation was determined 48 h later. $n = 6$ experiments, each condition assayed in triplicate. *$P <$ 0.01 vs. respective vehicle control. #$P < 0.01$ vs. A9/ET-1.

Discussion

In this manuscript, we identify a novel function of GSK-3β. We demonstrate that GSK-3β plays a critical role in the hypertrophic response of cardiomyocytes by showing that GSK-3β kinase activity is inhibited by hypertrophic stimuli both in vitro and in vivo, and that inhibition of GSK-3β activity is essential for all three components of the hypertrophic response of cardiomyocytes enhanced protein accumulation and sarcomere organization, and re-expression of fetal genes. Furthermore, we show that GSK-3β likely limits the hypertrophic response, at least in part, by negatively regulating ET-1–induced nuclear localization of NF-AT.

Numerous signaling molecules have been identified, which, when activated, transduce prohypertrophic signals, and some, such as the SAPK/JNKs, calcineurin, and the α subunit of Gq heterotrimeric G proteins, have been shown to be essential for the development of cardiac hypertrophy in vivo to physiologically relevant stimuli (reviewed in Force et al., 1999a,b; Olson and Molkentin, 1999; Sugden, 1999). To date, few studies have identified pathways that negatively regulate the hypertrophic response, yet these may be equally attractive targets for therapies designed to block the progression of hypertrophy and the transition to heart failure.

GSK-3 is normally active in unstimulated cells, and is inactivated in response to growth factors, especially insulin and IGF-1, which activate the PI3-K pathway (Avruch, 1998). We now demonstrate that GSK-3β is potently inhibited by hypertrophic agonists with receptors coupled to heterotrimeric G proteins of the Gq class. Morisco et al. (2000) recently showed that stimulation of β-adrenergic receptors, which are coupled to Gs proteins, also inhibited GSK-3β, suggesting that inhibition of this kinase may be a generalized phenomenon of hypertrophic signaling in cells in culture. We also showed that GSK-3β is markedly inhibited in the intact animal exposed to pressure overload induced by aortic banding, a stress that mimics severe valvular or hypertensive disease.

Recently, Rezvani and Liew (2000) reported that human hypertrophy is associated with elevated levels of β-catenin protein, a transcriptional activator involved in embryonic development and tumorigenesis, not previously known to play a role in hypertrophy. Since GSK-3β, when active, phosphorylates β-catenin, targeting it for ubiquitination and degradation, inhibition of GSK-3β may account, in part, for the increased expression of β-catenin. We believe β-catenin may be an additional target of GSK-3β that is involved in the hypertrophic response and studies evaluating this hypothesis are in progress.

GSK-3β activity can be inhibited by a number of mechanisms, but our findings suggest that hypertrophic agonists utilize phosphorylation of Ser 9 by a PI3-K–dependent protein kinase. This phosphorylation can be catalyzed by at least two protein kinases, PKB/Akt and ILK, both of which are activated by phosphatidylinositol 3 phosphates (Coffer et al., 1998; Delcommenne et al., 1998). It is unclear which of the two is the physiologically relevant kinase that inactivates GSK-3 in response to hypertrophic stimuli. The data confirm that the inhibition of GSK-3β is not mediated via recruitment of the Wnt pathway by hypertrophic agonists since Wnt-induced inhibition of GSK-3β appears to be PI3-K-independent (Cook et al., 1996).

Ultimately, cytosolic signaling pathways must modulate the activity of various transcription factors to direct the complex reprogramming of gene expression required to express the full hypertrophic phenotype. Our data suggest that one critical target of GSK-3 that likely plays a role in the hypertrophic response of cardiac myocytes is a member of the NF-AT family of transcription factors. NF-AT family members have been implicated in both cardiac hypertrophy and IGF-1–induced skeletal myocyte hypertrophy (Molkentin et al., 1998; Musaro et al., 1999). NF-AT activity is largely controlled at the level of nuclear localization since the cytoplasmic forms are competent for both DNA binding and transcriptional activation (Beals et al., 1997a,b). NF-ATs appear to be held in the cytoplasm by the masking of two nuclear localization signals by the intramolecular interaction of several phosphorylated serine residues with a second serine-rich region (Crabtree, 1999). Dephosphorylation of the serine residues by calcineurin exposes the nuclear localization signals leading to nuclear import.

Several protein kinases in addition to GSK-3β have been implicated in the nuclear export and/or cytosolic anchoring of NF-AT family members. Three MAP kinases (SAPKs, ERKs, and p38), protein kinase A, casein kinase Iα in cooperation with MAP kinase/ERK kinase-1, and casein kinase 2 have been reported to phosphorylate critical residues in the SerPro-rich domain of one or more NF-ATs, blocking nuclear import and/or enhancing export (Zhu et al., 1998; Chow et al., 2000; Porter et al., 2000). The role of these kinases has been examined primarily in T cells, hippocampal neurons, or transformed cell lines commonly

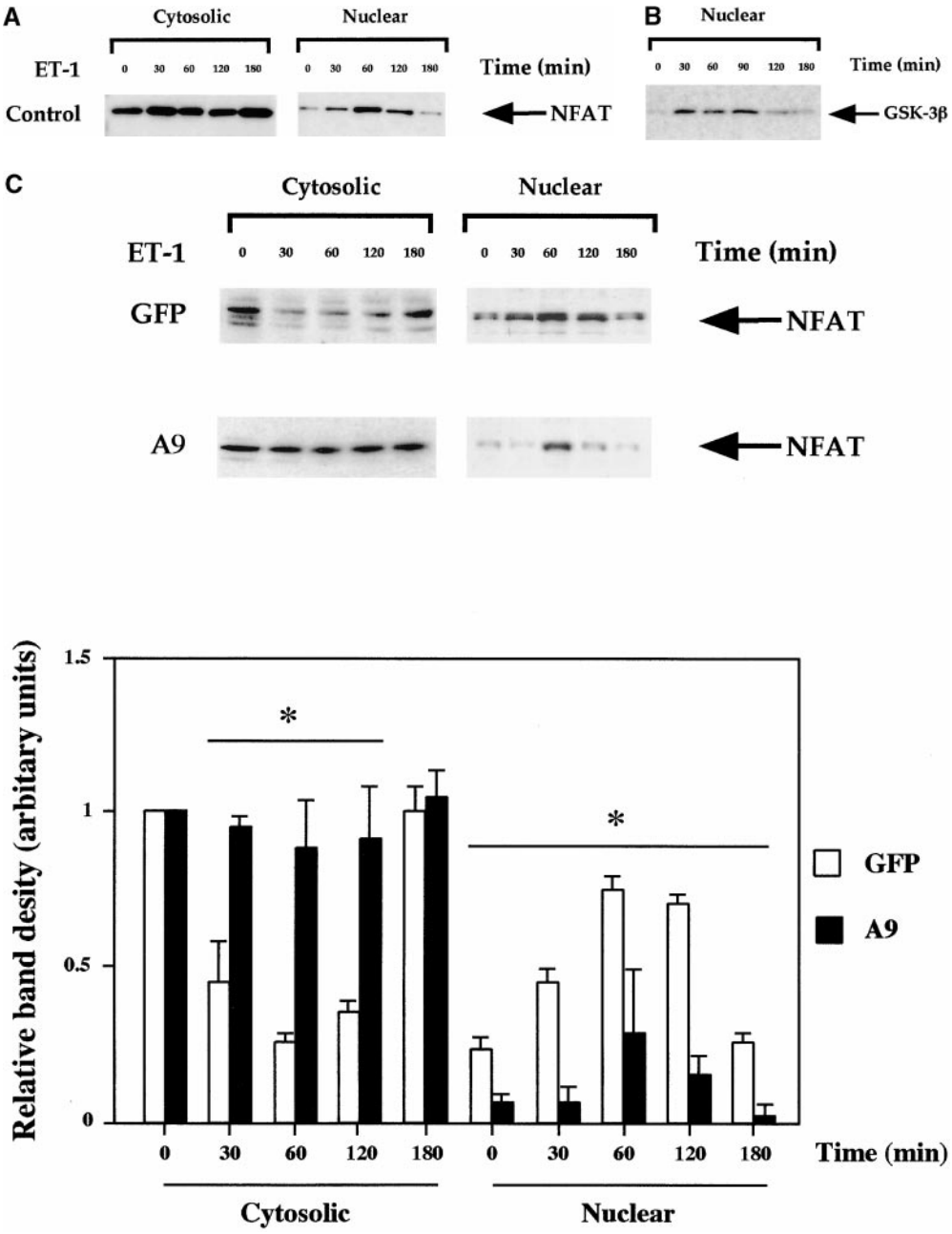

Figure 8. Effect of GSK-3βA9 on ET-1–induced nuclear translocation of NF-AT. A, ET-1 induces nuclear translocation of NF-AT. Cardiomyocytes were stimulated with ET-1 for the times noted. Cytosolic and nuclear fractions were prepared, matched for protein, and immunoblotted with anti–NF-AT antibody. This and all other immunoblots shown in this figure are representative of three experiments. B, ET-1 induces nuclear translocation of GSK-3β. Cells were stimulated with ET-1 for the times noted and then were subjected to cell fractionation. Nuclear fractions were matched for protein, and immunoblotted with anti–GSK-3β. C, Expression of GSK-3βA9 reduces ET-1–induced nuclear translocation of NF-AT. Cardiomyocytes were infected with AdGFP (top), or AdGSK-3βA9 (bottom), and 36 h later were stimulated with ET-1 for the times noted. Cytosolic and nuclear fractions were matched for protein, and immunoblotted with anti–NF-AT antibody. Below the immunoblots is a graph showing quantitation of NF-AT cytosolic and nuclear localization by band densitometry from $n = 3$ experiments. Each value is normalized to the respective cytosolic band density at 0 min. $^*P < 0.05$ for AdGSK-3βA9 infected cells vs. AdGFP infected cells.

used in studies using transfection (e.g., COS cells), and few studies have focused on the regulation of endogenous NF-ATs. It appears from these studies that the relevant kinase(s) regulating NF-AT nuclear/cytosolic partitioning depends on the NF-AT, the cell type, and, possibly, the stimulus. For our purposes, Beals et al. (1997b) have clearly shown that GSK-3 regulates nuclear export of NF-ATc1, the NF-AT we believe to be most highly expressed in neonatal cardiomyocytes. More recently, Porter et al. (2000) proposed that casein kinase 2, which is constitutively nuclear localized, may serve as a priming kinase that phosphorylates residues of NF-ATc1, allowing more efficient phosphorylation by GSK-3β. They noted however, that GSK-3β could, itself, also serve as the priming kinase.

Although the role of the other putative NF-AT kinases in cardiomyocytes is not clear, out data confirm a critical role for GSK-3β. We found that expression of GSK-3βA9 delayed the initial ET-1–induced import of NF-AT into the nucleus, and, subsequently, enhanced nuclear export, resulting in a markedly reduced duration of NF-AT nuclear localization. These data are compatible with an important role for GSK-3β in nuclear/cytoplasmic partitioning of NF-AT after stimulation by hypertrophic agonists. In addition, we found that treatment of cells with LiCl in the absence of ET-1 induced marked translocation of NF-AT to the nucleus (data not shown), suggesting that GSK-3β may not only retard stimulus-induced entry of NF-AT into the nucleus, but also may be the dominant mechanism for maintaining NF-AT in the cytosol in the unstimulated or resting cardiac myocyte.

An alternative approach to studying the role of GSK-3β (and NF-ATs) in the hypertrophic response would be to

create mice deleted for one or more of these genes. However, the GSK-3β deletion is embryonic lethal. Furthermore, the molecular mass of the dominant NF-AT in neonatal rat cardiomyocytes is most compatible with NF-ATc1, and mouse embryos lacking NF-ATc1 die at day 11 from congestive heart failure due to improper formation of the cardiac valves (Ranger et al., 1998). Mice deleted for the other NF-ATs expressed in the heart are viable, but since cardiac myocytes contain more than one NF-AT, deletion of one may be compensated for by the others. Cross-breeding viable knockouts could clarify the role of the NF-ATs in hypertrophy, but increases the probability of embryonic lethality.

Although we believe NF-ATs are important in the hypertrophic response, several pieces of evidence suggest that inhibition of NF-ATs is not the only mechanism by which GSK-3 attenuates the hypertrophic response. For example, in contrast to the marked hypertrophy seen when an activated mutant of NF-ATc4, NF-ATc4Δ317, is expressed in the hearts of transgenic mice (Molkentin et al., 1998), expression of NF-ATc4Δ317 in neonatal rat cardiomyocytes in culture induces a definite, but modest hypertrophic response. Therefore, either NF-ATs are necessary, but not sufficient for the full expression of the hypertrophic phenotype, or, more likely, there are additional targets activated by ET-1 and PE (and inhibited by GSK-3β) that play a role in the hypertrophic response. In support of the latter possibility, preliminary experiments suggest that expression of NF-ATc4Δ317 partially, but not completely, overcomes the inhibitory effect of GSK-3βA9 on the hypertrophic response. In this regard, GSK-3 has another target, c-Jun, which has been implicated in the hypertrophic response. c-Jun was the first transcription factor identified as a substrate of GSK-3 (de Groot et al., 1993; Nikolakaki et al., 1993). GSK-3 phosphorylates several residues near the DNA binding domain of c-Jun, and this negatively regulates the DNA binding activity of the transcription factor. We have previously shown that the SAPKs/JNKs, which increase the transcriptional activating activity of c-Jun, are necessary for the hypertrophic response of cardiomyocytes both in vitro and in vivo (Choukroun et al., 1998, 1999), and, given the number of hypertrophic response genes that appear to be regulated, at least in part, by AP-1, a heterodimer of c-Jun and c-Fos (reviewed in Olson and Molkentin, 1999), it is likely that c-Jun plays an important role in hypertrophy. Therefore, inhibition of c-Jun and, as a result, AP-1, may be a mechanism in addition to inhibition of NF-ATs, whereby GSK-3 signals to blunt the hypertrophic response. In addition to the effects of AP-1 itself on gene expression, AP-1 is also required for efficient binding of NF-ATs to DNA (Rao et al., 1997), suggesting an additional mechanism whereby GSK-3β, via inactivation of AP-1, could block NF-AT–dependent gene expression.

We employed LiCl to inhibit activity of GSK-3βA9 and endogenous GSK-3. LiCl has been employed to this end to study the role of GSK-3 in embryonic development in organisms as diverse as *Dictyostelium*, *Xenopus laevis*, sea urchins, and zebrafish, and to study numerous processes in mammalian cells (Klein and Melton, 1996; Stambolic et al., 1996; Yamamoto et al., 1999). LiCl has no known effects on other protein kinases, but does have effects on other enzymes, including inhibiting the inositol monophosphatase and adenylyl cyclase. Whereas LiCl may have other less well-described ancillary effects, the direct reversal by LiCl of the effects of GSK-3βA9 on sarcomere organization, ANF expression, and protein synthesis suggests Li^+ mediated its actions primarily via inhibition of GSK-3. An alternative strategy would have been to use kinase-inactive GSK-3β. In our hands and others, kinase-inactive GSK-3β is not an adequate dominant inhibitory mutant in mammalian cells (Yuan et al., 1999), and needs to be expressed at high levels to function as an inhibitor of GSK-3 signaling. This may lead to nonspecific effects and requires infection at high MOIs that can be toxic to cardiac myocytes. Whereas this might not adversely affect activity of reporter constructs (Morisco et al., 2000), it does disrupt the complex and highly coordinated responses required to produce the hypertrophic phenotype.

In summary, we have identified a novel role for GSK-3 as a critical negative modulator of cardiomyocyte hypertrophy. Our data suggest a model whereby GSK-3 directly antagonizes the prohypertrophic effects of activated calcineurin by inhibiting activity of one of its primary targets, NF-AT. The elucidation of a central role for GSK-3 in hypertrophy identifies not only GSK-3 and its downstream effectors, but also a large number of signaling molecules upstream of GSK-3, including PI3-Ks, polyphosphatidylinositide-dependent protein kinases (PDKs), PKB/Akt, and ILK, as potential therapeutic targets for drugs to alter the natural history of hypertrophy and heart failure.

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