Integrin-Linked Kinase Expression Is Elevated in Human Cardiac Hypertrophy and Induces Hypertrophy in Transgenic Mice

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***Background*—**Although numerous signaling pathways are known to be activated in experimental cardiac hypertrophy, the molecular basis of the hypertrophic response inherent in human heart diseases remains largely unknown. Integrin-linked kinase (ILK) is a multifunctional protein kinase that physically links β-integrins with the actin cytoskeleton, suggesting a potential mechanoreceptor role.

***Methods and Results*—**Here, we show a marked increase in ILK protein levels in hypertrophic ventricles of patients with congenital and acquired outflow tract obstruction. This increase in ILK was associated with activation of the Rho family guanine triphosphatases, Rac1 and Cdc42, and known hypertrophic signaling kinases, including extracellular signal-related kinases (ERK1/2) and p70 S6 kinase. Transgenic mice with cardiac-specific expression of a constitutively active ILK (ILK^{S343D}) or wild-type ILK (ILK^{WT}) exhibited a compensated ventricular hypertrophic phenotype and displayed an activation profile of guanine triphosphatases and downstream protein kinases concordant with that seen in human hypertrophy. In contrast, transgenic mice with cardiomyocyte-restricted expression of a kinase-inactive ILK (ILK^{R211A}) were unable to mount a compensatory hypertrophic response to angiotensin II in vivo.

***Conclusions*—**Taken together, these results identify ILK-regulated signaling as a broadly adaptive hypertrophic response mechanism relevant to a wide range of clinical heart disease. **(*Circulation*. 2006;114:2271-2279.)**

Key Words: angiotensin ■ hypertrophy ■ molecular biology ■ signal transduction

Ventricular hypertrophy is an extremely common clinical condition that appears as a consequence of any variety of volume- and or pressure-overload stresses on the human heart.[1,2] An increase in ventricular mass occurring in response to increased cardiac loading is generally viewed as a compensatory response that serves to normalize ventricular wall tension and improve pump function. Conversely, a sustained or excessive hypertrophic response typically is considered maladaptive on the basis of the progression to dilated cardiac failure sometimes observed clinically and the statistical association of ventricular hypertrophy with increased cardiac mortality.[3] Whereas mouse models of cardiac hypertrophy have been generated by genetically induced alterations in the activation state of various kinases in the heart,[4] limited information is available regarding the role of specific signaling pathways activated during human ventricular hypertrophy.

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In cardiomyocytes, integrins are thought to transduce biomechanical stress into a compensatory growth program on the basis of their role in linking the extracellular matrix with intracellular signaling pathways affecting growth and survival.[5] Integrin-linked kinase (ILK) is a protein serine/threonine (Ser/Thr) kinase that binds to the cytoplasmic domains of β1-, β2-, and β3-integrin subunits.[6] ILK serves as a molecular scaffold at sites of integrin-mediated adhesion, anchoring cytoskeletal actin and nucleating a supramolecular complex made up minimally of ILK, particularly interesting new cysteine-histidine–rich protein, and β-parvin.[7] In addition to playing a structural role, ILK is a signaling kinase coordinating cues from the extracellular matrix in a phosphoinositide 3′-kinase (PI3K)–dependent manner following distinct signal inputs from integrins and growth factor receptor tyrosine

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The online-only Data Supplement, consisting of expanded Methods, tables, and figures, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.106.642330/DC1.

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kinases.[8–10] ILK lies upstream of kinases shown in experimental models to modulate hypertrophy and in some cell types is required for phosphorylation of protein kinase B (Akt/PKB) at Ser473 and glycogen synthase kinase 3β (GSK3β) at Ser9.[11,12] Rho-family guanine triphosphatases (GTPases, or G proteins), including RhoA, Cdc42, and Rac1, modulate signal transduction pathways regulating actin cytoskeletal dynamics in response to matrix interaction with integrin and other cell surface receptors (reviewed elsewhere[13]). Both RhoA and Rac1 have been shown to modulate cardiac hypertrophy.[14,15] Extracellular matrix adhesion stimulates the increased association of activated GTP-bound Rac1 with the plasma membrane, suggesting a role for ILK in promoting membrane targeting of activated Rac1.[16] ILK thus links integrins with the force-generating actin cytoskeleton and is therefore a candidate molecule in the transduction of mechanical signals initiated by altered loading conditions affecting the heart.

Here, we demonstrate that ILK protein expression is increased in the hypertrophic human ventricle and find that ILK expression levels correlate with increased GTP loading, or activation, of the small G protein Rac1. Transgenic mice with cardiac-specific activation of ILK signaling are shown to exhibit compensated left ventricular (LV) hypertrophy. In agreement with the findings in the human hypertrophic heart, ventricular lysates derived from ILK-overexpressing mice lines, as well as isolated human cardiomyocytes, exhibit higher levels of activated Rac1 and Cdc42 in association with activation of p38 mitogen-activated protein kinase, extracellular signal–regulated kinase 1/2 (ERK1/2), and p70 S6 kinase (p70S6K) cascades. The transgenic (Tg) models indicate that ILK induces a program of prohypertrophic kinase activation and suggest that ILK represents a critical node linking increased hemodynamic loading to a cardioprotective, hypertrophic signaling hierarchy. Moreover, the ILK Tg mouse is shown to provide a new model of cardiac hypertrophy that is highly relevant to human cardiac disease.

Methods

The methods used to generate Tg mouse lines for invasive hemodynamic measurements, for Western blot analyses, and for incorporation of [^{35}S] methionine into isolated cardiomyocytes are given in the online Data Supplement.

Two-Dimensional Echocardiography

Serial 2-dimensional echocardiography was performed in male ILK Tg and nontransgenic (NTg) littermate mice at 10 to 12 weeks and 5 and 15 months of age. An ultrasound biomicroscope (VS40, VisualSonics Inc, Toronto, Ontario, Canada) with a transducer frequency of 30 MHz was used to make M-mode recordings of the left ventricle. Anterior and posterior LV free wall thickness and ventricular chamber dimensions were measured at end systole and end diastole; the contractility indexes, velocity of circumferential fiber shortening, percent fractional shortening, and LV ventricular mass were calculated as described.[17]

ILK Immune Complex Kinase Assay

Cells were lysed in NP40 buffer, supplemented with 1 mmol/L sodium orthovanadate and 5 mmol/L sodium fluoride as phosphatase inhibitors. Equal amounts of protein from these cell lysates were immunoprecipitated with anti-ILK polyclonal antibody, and immune complexes were incubated at 30°C for 30 minutes with myosin light chain II regulatory subunit (MLC_{20}) (2.5 μg per reaction) and γ[^{32}P] ATP (5 μCi per reaction) as previously described.[6]

Rho Family GTPase Activation Assays

Measurement of activated RhoA was performed with a pull-down assay based on specific binding of Rho-GTP to the Rho-binding domain of the Rho effector molecule rhoketin.[18] Cdc42 and Rac1 activation was measured with a pull-down assay based on the ability of the p21-binding domain of p21-associated kinase to affinity precipitate Rac1-GTP and Cdc42-GTP as described.[18]

Infusion of Angiotensin II

Pressor doses of angiotensin II (Ang II; 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or saline were administered for 4 weeks via osmotic minipumps (Alzet model 2004, Durect Corp, Cupertino, Calif) to Tg mice harboring the kinase-inactive, cardiac-restricted ILK (R211A) mutation or to NTg littermate controls as described.[19] The effects of Ang II on blood pressure were determined with a tail-cuff system (CODA 6, Kent Scientific, Torrington, Conn); hypertrophy was assessed by echocardiography and measurement of the ratio of heart weight to body weight; histological fibrosis was evaluated by Masson's trichrome staining; and reverse-transcription polymerase chain reaction (RT-PCR) was performed to detect atrial natriuretic factor and type I collagen message as described.[20]

Histopathology

The hearts were weighed, embedded in paraffin, sectioned at 1-mm intervals, and stained with hematoxylin and eosin and Sirius red using standard methods.[21] Micrographs were taken at both low ($\times$2.5) and high ($\times$40) magnification using fluorescent microscopy, and genotype-specific cardiomyocyte areas were determined from digital measurements of $>$500 cells per animal and 5 animals per genotype using Image J software (http://rsb.info.nih.gov/ij/).

Adenovirus-Mediated Expression of ILK Variants in Primary Cardiomyocytes

Human fetal cardiomyocytes (gestational age, 15 to 20 weeks) were obtained under an Institutional Review Board–approved protocol and cultured to $\approx$50% confluence (day 3 to 4 after plating) in preparation for adenovirally mediated infection of ILK constructs as previously described.[22,23] Replication-deficient serotype 5 adenovirus encoding the human wild-type ILK gene (Ad-ILK^{WT}) or kinase-inactive (Ad-ILK^{R211A}) or empty virus constructs previously shown to modulate ILK expression and activity in L6 myoblasts[24] were used for infection of human fetal cardiomyocytes. Human fetal cardiomyocytes were infected at 37°C at a multiplicity of infection of 2. KP392[25] and Kp307-2[26] are small-molecule inhibitors of ILK.

Human Ventricular Samples

All human tissue was acquired after protocol review and approval by the appropriate research ethics board, and the protocols were conducted in accordance with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans.

Statistical Methods

Determination of significant genotype-specific differences in 2-dimensional echocardiography and cardiac catheterization data relied on a paired t test or analysis of variance (ANOVA) in the case of serial measurements, followed by the multiple-comparison Bonferroni t test to assess differences among groups. The significance level was set at $P<0.05$.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

ILK Protein Levels Are Elevated in Human Cardiac Hypertrophy

To test for the participation of ILK in hypertrophic heart disease in vivo, we examined ILK expression in human

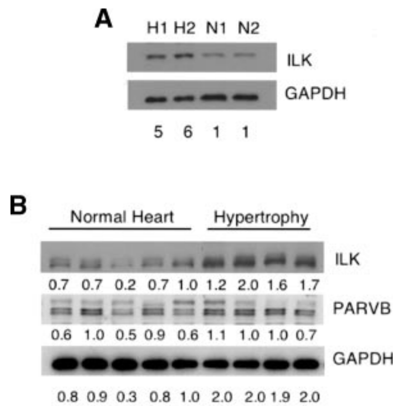

Figure 1. ILK expression in normal and hypertrophied human ventricles. A, Ventricular lysates from patients with congenital outflow tract obstruction (H1, H2) exhibiting severe hypertrophic valvular heart disease and from (nonhypertrophic) normal human fetal (19-week-old) ventricle (N1, N2) were immunoblotted for levels of ILK protein with glyceraldehyde phosphate dehydrogenase (GAPDH) as loading control. Ratios indicate ILK protein levels normalized to GAPDH. B, Ventricular lysates from hypertrophic (hypertrophic obstructive cardiomyopathy) and normal (nonhypertrophied) human hearts were analyzed by Western blotting for levels of ILK and ß-parvin. GAPDH was the loading control.

ventricular tissue samples from patients with and without clinically evident hypertrophy. Ventricular samples were acquired from 2 patients in the first year of life with ventricular hypertrophy secondary to congenital outflow tract obstruction; control ventricular tissue was derived from structurally normal, 19-week human fetal hearts (n=2) and examined in parallel for levels of ILK expression. Ventricular tissue from these hearts exhibited a 5- to 6-fold increase in ILK protein levels over control levels (Figure 1A).

We then investigated whether ILK protein expression was elevated in hypertrophy caused by LV outflow tract obstruction because clinical hypertrophic heart disease more commonly affects the left ventricle. Surgical specimens were acquired from the LV outflow tract in adult patients (n=4) with hypertrophic obstructive cardiomyopathy exhibiting resting LV outflow tract obstruction gradients >50 mm Hg. Control ventricular tissue was obtained from structurally normal hearts (n=5) at the time of multiorgan transplantation procurement. Myocardial samples from hypertrophic obstructive cardiomyopathy patients exhibited an ≈2-fold increase in ILK protein levels relative to control hearts (Figure 1B).

We also measured levels of β-parvin, a component of ILK multiprotein complexes. In contrast to ILK levels, β-parvin levels were not significantly different from those of controls, indicating hypertrophy-specific upregulation of ILK. Thus, the cases of clinical hypertrophy all demonstrated elevated ILK protein, suggesting that this is a critical molecular response to increased cardiac loading and the development of hypertrophy.

ILK has been shown to activate Rho family GTPases, which also have been causally implicated in experimental hypertrophy.[8] We therefore assayed the ventricular tissues directly for activation of RhoA, Cdc42, and Rac1 GTPases using specific affinity binding assays that distinguish the GDP-bound (inactive) and GTP-bound (active) states of each. Strikingly, there was an ≈2-fold and ≈10-fold increase in Rac1 GTP loading in the hypertrophic ventricular samples from patients with acquired and congenital outflow tract obstruction, respectively (Figure 2A and 2B). Cdc42 activation of ≈2-fold also was evident in both acquired and congenital hypertrophic lesions. Conversely, the levels of GTP-bound RhoA were unchanged between the control and hypertrophied ventricles. These results indicate selective activation of Rac1 and, to a lesser extent, Cdc42, coincident with increased ILK protein levels in human ventricular hypertrophy induced in both the left and right ventricles by obstructive hemodynamic loading.

Because the prohypertrophic kinases Akt/PKB, GSK3β, and ERK1/2 are known targets of ILK, we ascertained whether these proteins also were elevated in the cases of human hypertrophy. Western blotting for total protein indicated equivalent levels of GSK3β and ERK1/2 in the hypertrophied and normal hearts and an increase in PKB (Figure 3). We tested the hypertrophic hearts for concordant increases in the phosphorylation state of these targets of ILK that also have been implicated in the promotion of cardiac hypertrophy. Surprisingly, the phosphorylation state of Akt/PKB and GSK3β was not increased above control levels in any of the samples from the human hypertrophic ventricles (Figure 3A and 3B), despite the increased ILK protein levels in these samples. This result suggests that an ILK-Rac1 hypertrophic pathway is separable from ILK signaling through PKB/Akt and GSK3β. ERK1/2[27] and p70S6K,[9] which are kinases downstream of ILK, also have been implicated in the promotion of experimental cardiac hypertrophy in vivo. In contrast to Akt/PKB and GSK3β, ERK1/2 and p70S6K, but not p38

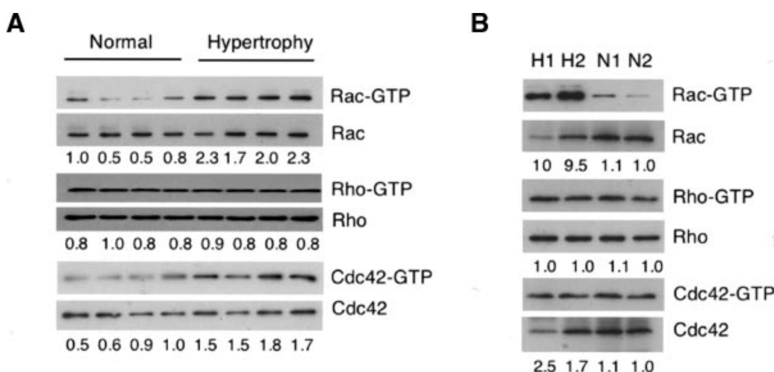

Figure 2. Rac, Rho, and Cdc42 expression in human heart tissue. A, Normal and hypertrophic human ventricular lysates were assayed for activation of Rho family GTPases as indicated. B, Ventricular lysates from the congenital samples (H1, H2) and normal human fetal (19-week-old) hearts (Figure 1) were assayed for Rho family activation. Ratios represent densitometric values of activated/total GTPase signals for Rho, Rac1, and Cdc42.

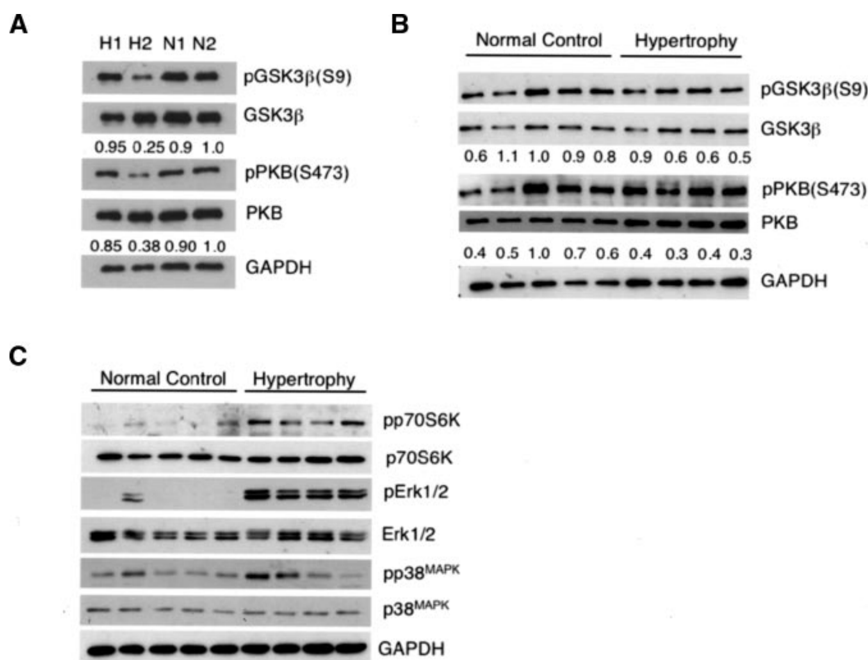

Figure 3. Phosphorylation of GSK3β, PKB, and MAP kinase in human heart tissue. Ventricular lysates from normal and hypertrophic human adult hearts were as in Figures 1 and 2. Lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blotting for levels of the indicated total and phosphorylated proteins.

mitogen-activated protein, were strongly phosphorylated in ventricular lysates in the setting of LV outflow tract obstruction (Figure 3C).

Cardiac-Specific Expression of Activated ILK in Transgenic Mice Induces Hypertrophy

The selective elevation of ILK levels in clinical cases of cardiac hypertrophy prompted us to ask whether increased ILK expression causes cardiac hypertrophy. To directly test hypertrophic responses to ILK in vivo, we derived independent lines of Tg mice harboring different ILK transgenes expressed under control of the cardiac-specific α-myosin heavy chain promoter. As discussed above, ILK is a multifunctional protein[9]; thus, our strategy was to generate lines expressing ILK variants that would allow us to differentiate kinase-dependent and -independent ILK functions in the heart. Toward this end, lines expressing constitutively activated ILK^{S343D}, ILK^{WT}, and ILK^{R211A} were derived.[28]

These data, presented in the online Data Supplement, show that cardiac-restricted ILK overexpression (ILK^{S343D} and ILK^{WT}) results in a compensated hypertrophic phenotype and that the capacity for hypertrophy induction is lost in the ILK loss-of-function mutation (ILK^{R211A}).

Induction of Cardiac Hypertrophy Is Dependent on the Activity of ILK

Our results showing hypertrophic induction by the activated ILK mutant and activity-dependent induction of ERK1/2 and p70S6K phosphorylation suggested that ILK-induced hypertrophy is dependent on ILK activity. To test this idea directly, we compared the hypertrophic status of hearts from Tg mice expressing ILK^{WT} with hearts from ILK^{R211A} Tg mice, which express a catalytically inactive variant of ILK. These data, presented in the online Data Supplement, indicate that the cardiac phenotypes of the various Tg mouse lines and the concordant changes in the phosphorylation state of ILK targets are dependent on the levels of ILK catalytic activity.

Ang II–Induced Hypertrophy Is Attenuated by ILK Loss of Function In Vivo

To test whether inhibiting ILK alters the cardiac remodeling response to a standard hypertrophic stimulus, pressor doses of Ang II ($2\ \mu g \cdot kg^{-1} \cdot min^{-1}$) or saline were administered for 4 weeks to Tg mice harboring the kinase-inactive, cardiac-restricted ILK (R211A) mutation, ILK^{R211A}, and to NTg littermate controls. As reported by others,[19,29] Ang II treatment resulted in increases in systolic and diastolic blood pressures ($P<0.01$ for all comparisons), which was similar in magnitude in Tg and NTg animals. Compared with NTg saline controls, NTg mice receiving Ang II exhibited concentric hypertrophy evident as a significant reduction in LV end-diastolic diameter and an increase in the ratio of heart weight to body weight and showed increased contractility evident as increased fractional shortening (the Table) ($P<0.05$ for all comparisons). Ang II–induced reduction in LV end-diastolic diameter and increased fractional shortening have been previously reported in wild-type mice.[30] Compared with NTg mice receiving Ang II, ILK^{R211A} mice treated with Ang II developed significantly less hypertrophy at 2 and 4 weeks as assessed by echocardiographic free wall thickness measurements ($P<0.01$, ANOVA) and by a reduction in the ratio of heart weight to body weight ($P<0.05$, ANOVA; the Table). Furthermore, ILK^{R211A} mice showed abrogation of the compensatory increase in contractility as measured by percent fractional shortening in response to Ang II observed in NTg vehicle controls.

As assessed by quantitative RT-PCR, NTg mice exhibited significant increases in message for collagen type III and atrial natriuretic peptide (ANP) relative to NTg controls in response to Ang II–induced hypertrophy (supplemental Figure III). In contrast, the catalytically inactive ILK mutant,

Ang II–Induced Hypertrophy Is Attenuated in ILK^{R211A} Transgenic Mice

	NTg Saline (n=7)	Tg Saline (n=8)	NTg + Ang II (n=7)	Tg+Ang II (n=9)
LVEDAW, mm				
Before Ang II	0.73±0.038	0.71±0.049	0.75±0.073	0.67±0.1
2 wk	0.73±0.07	0.75±0.086	0.96±0.12	0.81±0.12‡
4 wk	0.69±0.11	0.78±0.099	1.03±0.11*	0.77±0.08§
LVEDD, mm				
Before Ang II	3.95±0.23	4.1±0.26	4.0±0.17	3.98±0.11
2 wk	3.99±0.16	4.03±0.24	3.8±0.44	4.10±0.28§
4 wk	4.10±0.3	4.10±0.35	3.7±0.086*	4.10±0.18§
LVEDPW, mm				
Before Ang II	0.61±0.081	0.57±0.033	0.66±0.11	0.63±0.06
2 wk	0.65±0.058	0.70±0.092	0.91±0.15†	0.79±0.22
4 wk	0.67±0.21	0.72±0.16	1.0±0.092†	0.71±0.08§
FS, %				
Before Ang II	31±2.5	32±2.0	31±3.1	32±6.1
2 wk	33±2.2	32±2.7	42±2.4*	31±2.9
4 wk	31±3.5	33±2.8	41±3.6*	30±4.6
Heart weight, mg	121±5.7	124±7.8	181±9.5*	140±7.7‡
Body weight, g	25±0.8	25±0.6	25±1.2	24±0.6
Ratio of heart weight to body weight	4.8±0.15	4.9±0.32	7.1±1.4†	5.9±0.35‡

LVEDAW indicates LV end-diastolic anterior wall thickness; LVEDD, LV end-diastolic dimension; LVEDPW, LV end-diastolic posterior wall thickness; and FS, fractional shortening.

*$P<0.05$, †$P<0.01$, Tg saline vs NTg saline; ‡$P<0.05$, §$P<0.01$, Tg+Ang II vs NTg+Ang II mice.

ILK^{R211A}, showed no significant change in type III collagen or ANP message, which paralleled the blunted hypertrophic response to Ang II observed in this genotype (Figure 4).

Acute ILK-Dependent Rac1 Activation in Isolated Human Cardiomyocytes

To evaluate the effect of acute ILK upregulation on GTPase activation, we infected human fetal cardiomyocytes with adenoviruses expressing ILK (Ad-ILK) or an empty virus control. Infection with Ad-ILK stimulated an ≈3-fold increase in levels of GTP-bound Rac1 and an ≈7-fold increase in GTP-bound Cdc42 at 24 hours after infection (Figure 5). These stimulations were blocked by treatment of the Ad-ILK–infected cells with the small-molecule ILK inhibitor KP-392,[31] further suggesting that ILK kinase activity is required for activation of these small GTPases. Infection of the cardiomyocytes with empty adenovirus carrying no ILK

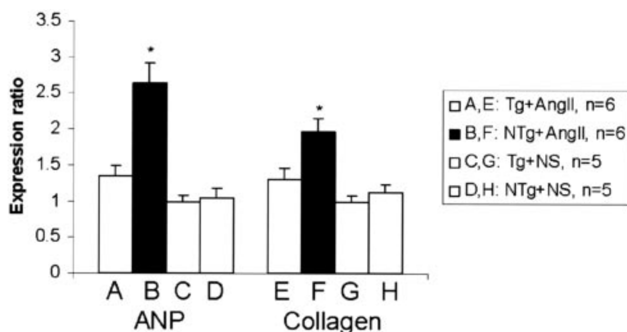

Figure 4. Ang II–induced changes in ANP and type I collagen. ANP and type III collagen expression was determined by RT-PCR in heart tissue of Tg (ILK^{R211A}) mice and NTg mice treated with either normal saline (NS) or Ang II. ANP and collagen values were normalized to GAPDH. $P<0.05$, NTg plus Ang II vs all others.

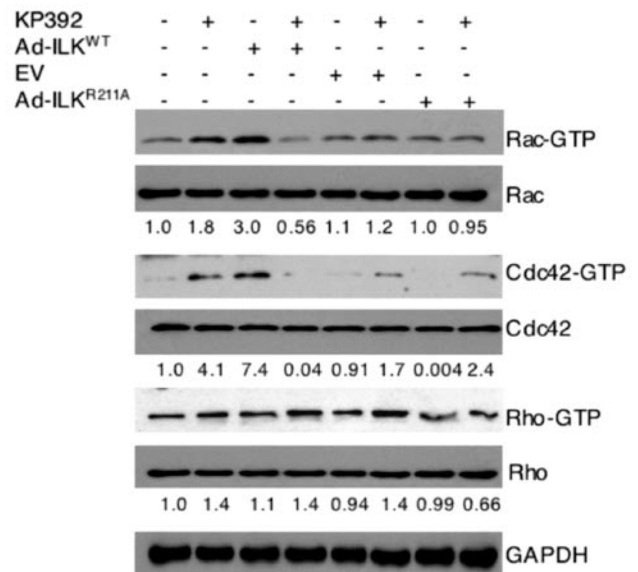

Figure 5. Selective activation of Rho family GTPases by ILK^{WT} but not ILK^{R211A} in primary human cardiomyocytes. Primary human fetal cardiomyocytes were infected with adenoviruses with or without (empty virus [EV]) ILK^{WT} or ILK^{R211A} cDNA. At 48 hours after infection, cells were harvested and lysates were assayed for activation of Rho family GTPases. As indicated, cultures were infected in the presence of the small-molecule ILK inhibitor KP-392.

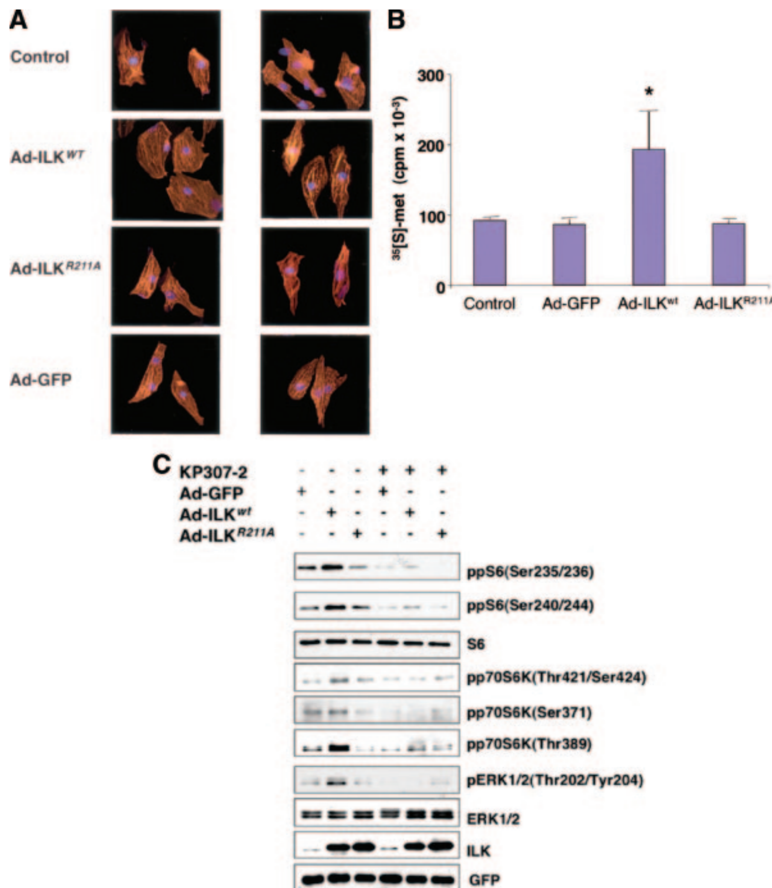

Figure 6. ILK induces hypertrophy in primary human fetal cardiomyocytes. A, Cells were infected with wild-type ILK (Ad-ILK^{WT}), mutant ILK (Ad-ILK^{R211A}), virus vector (Ad-GFP), or no virus (control) for 36 hours. Cells were treated with or without Kp307-2 for 12 hours before α-actinin staining. Expression of wild-type ILK, as opposed to expression of GFP or mutant ILK, markedly increases cell size and the extent of sarcomere organization; this effect of ILK is reversed by Kp307-2, an ILK inhibitor. B, [^{35}S] methionine (^{35}[S]-met)incorporation was highest in Ad-ILK^{WT}–treated cells ($P<0.05$, n=6). C, ILK-dependent activation of p70S6K. Phosphorylation of ERK1/2, p70S6K, and S6 protein was measured by Western blot in human fetal cardiomyocytes infected with adenovirus containing Ad-ILK^{WT} and Ad-ILK^{R211A} or Ad-GFP. Cells were treated with or without 5 μmol/L Kp307-2, a synthetic inhibitor of ILK, for 12 hours.

had no effect on the activation state of Rac1, Cdc42, or RhoA. These results indicate that, as in the Tg mouse hearts and during human hypertrophy caused by mechanical loading, acute upregulation of ILK in isolated cardiomyocytes directly activates Rac1 and Cdc42.

Acute ILK Activation Causes Hypertrophy in Isolated Human Cardiomyocytes

To evaluate the effect of acute ILK upregulation on hypertrophy induction, we infected human fetal cardiomyocytes with adenoviruses expressing wild-type ILK (Ad-ILK), mutant ILK (Ad-ILK^{R211A}), virus vector (Ad-GFP), or no virus (control) for 36 hours. Expression of wild-type ILK, as opposed to expression of GFP or mutant ILK, markedly increased cell size and the extent of sarcomere organization (Figure 6A) and is supported by an $\approx$2 fold ILK-dependent increase in protein synthesis as measured by [^{35}S] methionine incorporation (Figure 6B). These prohypertrophic effects were reversed by KP307-2, an ILK inhibitor.

ILK Activates the p70S6K Pathway in Isolated Human Cardiomyocytes

Ribosomal S6 protein is a component of the 40S subunit of the ribosome, which enhances protein translation by promoting ribosomal/mRNA association in response to activating phosphorylation mediated by p70S6K.[32] Activation of p70S6K itself occurs by hierarchical phosphorylation at key regulatory sites, including the mammalian target of rapamycin (mTOR) sites, Ser371 and Thr389, and ERK1/2-specific sites, Thr421 and Ser424. Adenoviral overexpression of wild-type ILK, but not the ILK^{R211A} mutant construct or GFP control, caused activation of p70S6K at Ser371 and Thr389, which was prevented by the small-molecule ILK inhibitor KP307-2[26] (Figure 6C). Because Thr421 and Ser424 are targets of ERK1/2,[33] we tested whether phosphorylation at these sites was ILK dependent. In concordance with the findings in the Tg mouse hearts (supplementary Figure IIIc and IIId) and human hypertrophic hearts (Figure 3C), our results indicate that acute ILK upregulation in human fetal cardiomyocytes in vitro also induces KP307-2–inhibitable phosphorylation of p70S6K; specifically, ILK activation resulted in an increased intensity of phospho-Thr421/Ser424 and phospho-Thr389 of p70S6K, indicating cooperative regulation by ERK1/2 and mTOR kinases, respectively (Figure 6C). Further evidence in support of ILK-dependent activation of p70S6K was the finding of increased ILK-specific phosphorylation of S6 ribosomal protein, which is a downstream target of p70S6K (Figure 6C).

Discussion

Principal Findings

Protein kinases are increasingly understood to be important regulators of cardiac hypertrophy; however, the critical question arises of whether kinases known to induce experimental hypertrophy are, in fact, upregulated or activated as a feature of human cardiac hypertrophy. To the best of our knowledge, our study is the first to unequivocally demonstrate increased

expression and activity of a candidate mechanotransducer, namely ILK, in human cardiac hypertrophy. Moreover, we show that moderate upregulation of ILK in the myocardium of Tg mice causes a compensated form of cardiac hypertrophy, as evidenced by preserved systolic and diastolic function, absence of histopathological fibrosis, and normal lifespan. Among a number of hypertrophy-inducing protein kinases that we examined, only 2, ILK and PKB, demonstrated elevated protein levels in association with hypertrophy. Of these, ILK was consistently elevated in both congenital and acquired hypertrophy. Two independent Tg mice lines harboring cardiomyocyte-restricted overexpression of ILK exhibited a profile of protein kinase activation strikingly similar to that seen in human cardiac hypertrophy. The fact that ILK upregulation is associated with mechanical load-induced hypertrophy (secondary to congenital and acquired forms of outflow tract obstruction) in which global cardiac function was preserved provides compelling evidence that ILK activation is associated with a provokable, compensatory form of hypertrophy in the human heart. We also show that hypertrophy associated with hypertension induced by Ang II is attenuated in Tg mice harboring the catalytic ILK mutant. Abrogation of the compensatory increase in contractility (observed in control Ang II–treated animals) in ILK mutant mice further reinforces the thesis that ILK is involved in a beneficial cardiac remodeling response. Our results showing a blunted hypertrophic response to Ang II in the ILK loss-of-function mutation are also consistent with the finding that Ang II was shown to activate cardiomyocyte Rac1.[34] The potential exists that the kinase-inactive ILK mutant does not cause true loss of function and may sequester another positive regulator of the hypertrophic response to Ang II, which may act independently of or in concert with ILK. Taken together, our human and mouse data suggest that ILK is a proximal mechanotransducer, acting to coordinate a program of downstream hypertrophic signal transduction in response to pressure overload in the myocardium.

Downstream Pathways Activated by ILK-Induced Hypertrophy

The lack of ILK-induced Akt/PKB and GSK3β phosphorylation in ILK Tg mice was unexpected, given that ILK is regulated in a PI3K-dependent manner and has been shown to directly phosphorylate both target kinases in noncardiomyocytes,[6,8,9,11] and contrasts with findings from genetic models of cardiac-specific PI3K and Akt/PKB activation, which feature increased phosphorylation of both Akt/PKB and GSK3β in proportion to the degree of hypertrophy.[35,36] We note, however, that levels of PKB Ser473 and GSK-3β Ser9 phosphorylation are quite high in both murine and human control hearts, consistent with the requirement for a threshold basal level of activation of these kinases, which may permit the induction of ILK-mediated hypertrophic signaling. Thus, our results are consistent with the operation of a p110α/ILK/Rac1 pathway but suggest that the ILK-specific hypertrophy is not critically dependent on increased phosphorylation of PKB/Akt or GSK3β. The relative deactivation of Akt/PKB during ILK transgenesis is consistent with the finding that activation of Akt/PKB and inhibitory phosphorylation of GSK3β occur in advanced failure but not during compensated hypertrophy in human hearts.[37] Thus, the lack of highly activated Akt/PKB in murine and human hearts exhibiting elevated ILK expression may be a signature of compensated hypertrophy.

ILK-Induced Hypertrophy Features Activation of Rac1 and p70S6K

Our results in Tg mice with ILK overexpression and in human hypertrophy and isolated human cardiomyocytes concordantly point to the selective activation of p70S6K as an important signal transducer element mediating ILK-driven myocyte hypertrophy. S6 kinases promote protein translation by phosphorylating the S6 protein of small ribosomal subunits and are required for mTOR-dependent muscle cell growth.[38] Activation of p70S6K can occur by dual activation of an mTOR-dependent pathway targeting Thr389 and an ERK1/2-dependent pathway targeting Thr421/Ser424, both of which are independent of the Akt/PKB pathway.[32,33] Further evidence in support of ILK-dependent activation of p70S6K was the finding in isolated cardiomyocytes of increased ILK-specific phosphorylation of S6 ribosomal protein, which is a downstream target of p70S6K. Our results extend the recent finding that the tripartite complex of ILK and the cytoskeletal interacting proteins particularly interesting new cysteine-histidine–rich protein and α-parvin is necessary for phenylalanine-induced hypertrophy in neonatal rat cardiomyocytes[39] and are consistent with the physiological hypertrophic response and augmented cardiac function associated with genetic stimulation of the ERK1/2 branch of the mitogen-activated protein kinase signaling pathway.[40] Considered together, our results indicate conservation of downstream signaling specificity resulting from ILK activation in both murine and human hypertrophy, which features activation of Rac1- and p70S6K-mediated signaling. Full elucidation of the unique network of effectors induced during ILK gain of function requires application of high-throughput functional proteomic approaches to genetic models and to stage-specific human diseases characterized by hypertrophic remodeling.

The reciprocal pattern of activation of Rac1 and deactivation of Rho has precedents and reflects opposing effects of these monomeric GTPases on the cytoskeleton at the leading edge of migrating cells.[41] Similarly, our results show reciprocal effects both in vitro and in vivo on the activation of Rac1/Cdc42 and Rho in response to ILK upregulation. These data are thus consistent with the observation that Tg mice overexpressing RhoA develop a predominantly dilated cardiomyopathic phenotype[42] that is antithetical to that observed with ILK activation. Our results showing a compensatory form of hypertrophy associated with activation of Rac1 also fit with the conclusion from a recent review that activation of RhoA and its principal effector kinase Rho-associated coiled-coiled protein kinase is generally deleterious in various models of myocardial ischemia-reperfusion, infarction, and congestive heart failure.[34]

Implications

In summary, our results identify a novel role for ILK-regulated signaling in mediating a broadly adaptive form of

cardiac hypertrophy. The effects of small-molecule inhibitors of ILK demonstrated experimentally[43] suggest that this pathway is therapeutically tractable and, together with our results, that modulation of the ILK pathway warrants evaluation as a novel approach to enhance the remodeling process relevant to a wide range of cardiac diseases.

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Disclosures

None.

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CLINICAL PERSPECTIVE

This study identifies a novel role for the multifunctional serine/threonine kinase, integrin-linked kinase (ILK), in the process of cardiac hypertrophy induction, which is notable because it appears to be relevant to human cardiovascular disease. ILK has emerged as an important kinase target in the processes of transformation and oncogenesis in many cell types. However, increased ILK expression in the heart, an organ notoriously resistant to transformation, seems to lead to a hypertrophic phenotype. Although the distinction between compensatory and maladaptive hypertrophic responses remains a clinically urgent question in the field, our results suggest that ILK activation is a signature of a compensatory type of response. This inference is based on the findings of elevated expression levels of ILK in distinct examples of apparently adaptive human hypertrophic responses and is reinforced by the concordance of phenotype and activation profile of signaling cascades found in transgenic mouse lines exhibiting variable degrees of ILK activation. Viewed this way, activation of target kinase signaling pathways modulated by ILK offers a theoretical but testable strategy for selectively boosting the adaptive response to chronic hypertrophic stimuli at the cardiomyocyte level. This approach should be potentially useful even after the transition to dilated heart failure characteristic of the advanced stages of a wide range of cardiovascular diseases.