Activation of pyk2/Related Focal Adhesion Tyrosine Kinase and Focal Adhesion Kinase in Cardiac Remodeling*

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Cellular remodeling during progression of dilation involves focal adhesion contact reorganization. However, the signaling mechanisms and structural consequences leading to impaired cardiomyocyte adhesion are poorly defined. These events were studied in tropomodulin-overexpressing transgenic mice that develop dilated cardiomyopathy associated with chronic elevation of intracellular calcium. Analysis of tropomodulin-overexpressing transgenic hearts by immunoblot and confocal microscopy revealed activation and redistribution of signaling molecules known to regulate adhesion. Calcium-dependent pyk2/related focal adhesion tyrosine kinase (RAFTK) showed changes in expression and phosphorylation state, similar to changes observed for a related downstream target molecule of pyk2/RAFTK termed focal adhesion kinase. Paxillin, the target substrate molecule for focal adhesion kinase phosphorylation, was redistributed in tropomodulin-overexpressing transgenic hearts with enhanced paxillin phosphorylation and cleavage. Certain aspects of the in vivo signaling phenotype including increased paxillin phosphorylation could be recapitulated in vitro using neonatal rat cardiomyocytes infected with recombinant adenovirus to overexpress tropomodulin. In addition, increasing intracellular calcium levels with ionomycin induced pyk2/RAFTK phosphorylation, and adenosylated mediated expression of wild-type pyk2/RAFTK resulted in increased phospho-pyk2/RAFTK levels and concomitant paxillin phosphorylation. Collectively, these results delineate a cardiomyocyte signaling pathway associated with dilation that has potential relevance for cardiac remodeling, focal adhesion reorganization, and loss of contractility.

Altered intracellular calcium handling is characteristic of heart failure, presumably as a compensatory mechanism to stimulate contractile function and signal transduction (1, 2). Chronic elevation of diastolic calcium level consequently activates calcium-dependent signal transduction pathways. Calcium-activated pathways regulate cardiac function, and disruption of calcium-responsive signaling has been associated with cardiomyopathic changes in experimental transgenic mouse models. Myocardium-specific transgenic overexpression of calcium-activated enzymes such as protein kinase C β2 (3, 4) or calcineurin (5) cause hypertrophy. Calcineurin activation, hypertrophic remodeling, and elevation of intracellular calcium are associated with heart failure in a cardiomyopathic transgenic mouse model created by overexpression of tropomodulin (Tmod),1 a regulatory component of the sarcomere (6). Tmod-overexpressing transgenic (TOT) hearts are characterized by a combination of increased intracellular calcium levels, myofibril degeneration, loss of systolic function, and inability to hypertrophy. This constellation of features is reminiscent of degenerative changes associated with heart failure (1, 7, 8), making the TOT mouse a useful model for studying structural and signaling changes occurring in myocardial decompensation. TOT hearts dilate rapidly between postnatal days 9 and 12, increasing heart:body weight ratio ~3-fold (6). This profound enlargement requires substantial cardiomyocyte remodeling driven, in part, by signaling pathways regulating cytoskeletal organization. Activation of cytoskeletal reorganization likely contributes to development of dilation in TOT hearts, as marked changes in cardiomyocyte shape occur (9) without the induction of typical genetic molecular markers usually associated with hypertrophic reprogramming (10). Cytoskeletal remodeling coupled with elevation of intracellular calcium level is associated with pyk2/RAFTK (abbreviations for proline-rich tyrosine kinase 2 and related focal adhesion tyrosine kinase, respectively; also known as CADTK, CAKδ, or FAK2), a cytosolic calcium-dependent tyrosine kinase with high homology to p125 focal adhesion kinase (FAK; Refs. 11 and 12). Elevation of intracellular calcium in TOT cardiomyocytes makes pyk2/RAFTK an ideal candidate molecule for initiating reorganization of focal adhesion contacts during the pathogenesis of dilation. Focal adhesions are macromolecular cytoskeletal structures that interface with integrins and are enriched for many molecules that serve roles in cytoskeletal architecture, signal transduction, or both. Although the pyk2/RAFTK expression and mechanisms of regulation have been extensively characterized in a variety of non-muscle (11, 12) and smooth muscle (13) cells and tissues, pyk2/RAFTK activity in normal or pathologically altered myocardium remains largely unexplored.

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1 The abbreviations used are: Tmod, tropomodulin; TOT, tropomodulin-overexpressing transgenic; NTG, nontransgenic; RAFTK, related focal adhesion tyrosine kinase; FAK, focal adhesion kinase; PBS, phosphate-buffered saline; WT, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; β-gal, β-galactosidase.
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Exploration. Recent studies from Samarel and co-workers (14) found pyk2/RAFTK concentration is much greater in neonatal than in adult ventricular tissue and cardiomyocytes, and pyk2/RAFTK expression is highly dependent on calcium signal transduction and contractility. In addition, pyk2/RAFTK expression level and phosphorylation were increased in a rat model of pressure overload cardiomyopathy (15). Both pyk2/RAFTK and FAK appear to exert their cytoskeletal signaling effects through phosphorylation of paxillin, a multifunctional adapter molecule concentrated at focal adhesion structures (16). Regulation of adhesion and cell migration has significant implications for cardiac function, because efficient force transmission depends in large part upon the integrity of connections between cardiomyocytes and the extracellular matrix.

Structural reorganization, loss of contractility, and elevation of intracellular calcium in juvenile TOT hearts presumably combine to initiate focal adhesion complex remodeling in the dilating heart. To test this hypothesis, TOT hearts and primary cardiomyocyte cultures were examined for activation of pyk2/RAFTK and associated downstream signaling molecules involved in regulation of focal adhesion complexes.

EXPERIMENTAL PROCEDURES

Mice—TOT mouse line and non-transgenic mice used in this study were created and bred as previously described (3) and handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Antibodies and Chemical Reagents—Phosphorylated sites on signaling proteins were detected using antibodies to pyk2/RAFTK (residue 402), FAK (residue 861), and paxillin (residue 31; all from BIO-SOURCE-QCB, Camarillo, CA). Signaling molecules were also labeled using anti-pyk2/RAFTK and anti-paxillin monoclonal antibodies (both from Transduction Laboratories, Lexington, KY) or anti-PAK polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY). Structural proteins were labeled with anti-vinculin (Santa Cruz Technologies, Santa Cruz, CA) and anti-a-actinin (Sigma). Nuclei were labeled with propidium iodide (Molecular Probes, Eugene, OR). Primary antibodies were detected by fluorescently tagged secondary goat anti-mouse IgG, donkey anti-goat IgG, and goat anti-rabbit IgG (Jackson Immunoresearch Laboratory, West Grove, PA). Juncycin was obtained from Sigma. Cardiomyocytes were treated with 0.5 μM juncycin for 25 min, washed twice in PBS, and processed to obtain a whole cell extract as described below.

Immunohistochemistry—Sections were prepared from TOT and control mice hearts, which were fixed in 4% paraformaldehyde/PBS over- night at 4°C. The next day, hearts were subjected to a progressive sucrose gradient from 10, 20, and 30% at 4°C for 24 hours at 4°C for 24 h to a multiplicity of infection of 50:1, and then medium was aspirated and replaced with maintenance medium. Lysates for biochemical analyses were prepared by homogenization in cell lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1% EGTA) in the presence of a mixture of protease (10 μM sodium orthovanadate and NaF, 10 μM sodium pyrophosphate, 1 mM dithiothreitol, and 10 μM CaCl2) and phosphatase (10 μM okadaic acid and 100 mM phenylsulfonyl fluoride) inhibitors. The homogenates were centrifuged at 15,000 g at 4°C for 20 min to obtain clarified lysates. For preparing lysates from cultured cells, cardiomyocytes were washed twice with PBS and lysed in the same extraction buffer used for heart homogenization and centrifuged in an identical manner prior to use in experiments. Tissue extracts and whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were probed with various primary antibodies as indicated and detected by enhanced chemiluminescence according to manufacturer protocols (Amersham Biosciences, Buckinghamshire, UK).

In Vitro Kinase Assays—The immunoprecipitated complexes, obtained by immunoprecipitating nontransgenic (NTG) hearts and TOT heart lysates with anti-RAFTK antibodies (1 mg/ml), were washed three times with lysis buffer and twice in kinase buffer (20 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 1 mM NaVO4, and 20 μM ATP). The kinase assay was initiated by incubating the immune complex in kinase buffer containing 25 μg of poly(Glu/Tyr) (4:1; 20–50 kDa; Sigma) and 5 μCi of [γ-32P]ATP at room temperature for 30 min. Reactions were terminated and analyzed as described (18–20).

Quantitation and Data Analysis—Each band was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal intensity. Results are expressed as means ± S.E. of the mean. Student’s t test was used for statistical comparisons. Differences among means were considered significant at p < 0.01.

RESULTS

pyk2/RAFTK Protein Shows Increased Expression and Activation in TOT Hearts—Hearts from nontransgenic control and TOT mice were compared by immunoblot analysis to determine total pyk2/RAFTK protein content. Labeling of SDS-PAGE separated lysates showed the pyk2/RAFTK content of TOT hearts was increased 2.4-fold compared with control animals (Fig. 1). Repeated experiments using multiple heart lysates from individual TOTs showed pyk2/RAFTK levels were consistently elevated in samples from TOTs with high heart/body weight (mg/kg) ratios (over 10:1). Phosphorylation of pyk2/RAFTK was detected using antibody that recognized phosphorytrosine at position 402 (phospho-pyk402), indicative of activation (11). Phospho-pyk2/RAFTK immunoactivity was increased 1.8-fold in TOT samples versus normal controls (Fig. 1). Thus, pyk2/RAFTK accumulation and activation is associated with TOT cardiac pathogenesis.

pyk2/RAFTK Translocation in TOT Hearts—Confocal microscopy was performed to identify phospho-pyk2/RAFTK102 signaling in histologic sections from TOT hearts and controls (Fig. 1). Experimental performed with anti-phospho-pyk102 (data not shown) yielded results comparable with findings with anti-phospho-pyk102. Analysis of sections from control nontransgenic hearts showed minimal reactivity with anti-phos-pyk102 (Fig. 1, A and C), consistent with immunoblot and previous results indicating low pyk2/RAFTK protein level in these hearts. In contrast, TOT sections showed marked phospho-pyk102 labeling, often in a perinuclear distribution (Fig. 1, B and D, arrow). As has been previously observed for pyk2/RAFTK immunolocalization (21). Closer examination revealed that activation of pyk2/RAFTK was focused in cardiomyocytes showing an increased anti-phospho-pyk102 labeling relative to...
pyk2/RAFTK (control) and TOT heart lysates. Immunoreactivity for both total pyk2/RAFTK and phospho-pyk2/RAFTK in TOT section at low or high magnification (red arrowheads, inset) is enriched in threads of non-uniform labeling, which appeared to lie between adjacent cells (Fig. 2B). Many areas of phospho-FAK (Fig. 2A) reactivity in TOT sections was enriched in threads of non-uniform labeling, which appeared to lie between adjacent cells (Fig. 2B).

Many areas of phospho-FAK (Fig. 2A) reactivity in the TOT sections colocalized with focal adhesion complexes identified with antibody to paxillin (Fig. 2, C and D, at arrows). Specificity of the anti-phospho-FAK antibody was demonstrated by peptide competition experiments (Fig. 2, E–H). As pyk2/RAFTK distribution, redistribution of activated FAK was associated to cardiomyocytes of TOT hearts, where the activated kinase may be targeted to focal adhesion complexes.

**pyk2/RAFTK and FAK Activity Are Increased in TOT Hearts**—Results indicating activation of pyk2/RAFTK and FAK in TOT hearts (Figs. 1 and 2) was confirmed by *in vitro* kinase assay (Fig. 3). Lysates from individual nontransgenic control (*n* = 5) or TOT (*n* = 5) hearts were subjected to immunoprecipitation with antibodies directed against either pyk2/RAFTK or FAK. Phosphorylation of target substrate by the immunoprecipitated kinase was quantitated, and -fold increases were calculated relative to immunoprecipitation with control irrelevant antibody. Results show a 4.1 ± 0.24-fold increase in pyk2/RAFTK activity and a 2.5 ± 0.24-fold increase in FAK activity relative to the comparably prepared nontransgenic samples. Kinase activity differences between the TOT and the comparably prepared nontransgenic samples were highly significant (*p* < 0.005). These results confirm elevation of pyk2/RAFTK kinase activity in the remodeling TOT myocardium.

**Paxillin Shows Enhanced Phosphorylation and Redistribution in TOT Hearts**—Paxillin is a direct phosphorylation target of FAK and pyk2/RAFTK that participates in regulation of focal adhesion structure based, in part, upon phosphorylation state (22). Phosphorylation of paxillin was assessed in TOT hearts using antibody against phosphotyrosine residue 31 (phospho-paxillin) because this residue is a target for FAK-mediated phosphorylation (24). The location of paxillin on the blot at the appropriate mobility of 68 kDa was visualized by using a positive control signal from a cultured neonatal rat cardiomyocyte lysate that overexpressed adenovirally encoded paxillin (Fig. 4, left side). Immunoblot analysis of lysates with anti-paxillin antibody showed a significant increase of 2.9-fold in phospho-FAK in TOT mice compared with nontransgenic animals (Fig. 2). This increase was also correlated with a change in phospho-FAK* in* distribution in same samples. Rather than the patchy, diffuse pattern observed in control sections (Fig. 2A), phospho-FAK* in* reactivity in TOT sections was enriched in threads of non-uniform labeling, which appeared to lie between adjacent cells (Fig. 2B).

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Fig. 1. pyk2/RAFTK protein level and activation are increased in TOT hearts. Top, representative immunoblots with antibodies to pyk2/RAFTK and phospho-pyk2/RAFTK using nontransgenic control (control) and TOT heart lysates. Immunoreactivity for both total pyk2/RAFTK (top row) and phospho-pyk2/RAFTK (middle row) is increased in TOT heart sample relative to nontransgenic control sample. Position of the pyk2/RAFTK band on each blot is indicated (arrow), and -fold increases in signal are shown to the right of each lane relative to the control sample. Calculated -fold increases between samples are corrected for minor variations in loading by standardization to GAPDH signals from the same blot (bottom row). Significant values were determined from a minimum of three different samples from separate experiments for each analysis. Bottom, sections (A–D) and cardiomyocytes (E–G) from nontransgenic control (A, C, and E) and TOT (B, D, F, and G) hearts labeled with antibody to phospho-pyk2/RAFTK (green). Cardiomyocytes are identified with antibody to α-actinin (blue). Nuclei are shown as landmarks of cell organization (red). Phospho-pyk2/RAFTK labeling is absent from control section at low or high magnification (A and C, respectively). In contrast, marked pyk2/RAFTK immunoreactivity is present in TOT section in a widespread distribution (B). High magnification reveals phospho-pyk2/RAFTK labeling is predominantly localized near nuclei in sections (D, arrow). Cardiomyocyte from control heart shows lack of phospho-pyk2/RAFTK labeling (E), whereas cardiomyocytes from TOT hearts show pyk2/RAFTK labeling in a striated pattern localized in the I band region (F, inset; pyk2/RAFTK bands indicated by brackets under arrows). Z-disc distribution in cardiomyocytes is shown by labeling with antibody to α-actinin (blue; arrowheads in F, inset). The perinuclear staining observed in the sections was much more intense than the relatively faint sarcomeric patterns shown in the isolated cell; hence, they appear as the dominant feature in the sections.
control cell lysate (+) and TOT samples relative to nontransgenic hearts (Fig. 4, right side, p-fold increase = 1.8 ± 0.2, p = 0.01). This polypeptide, which also labels with the pan-paxillin antibody (Fig. 4, left side), probably represents a cleaved paxillin fragment (25, 26) in the failing heart. Another related phosphopeptide with higher mobility (~50 kDa) was detected in the heart lysates but not in the positive control (+) sample (Fig. 4, right side), so the significance of this cross-reactive phosphopeptide remains unclear although immunoreactivity parallels the increases observed for the 38-kDa phospho-paxillin31 fragment. Confocal analysis showed that distribution...
of phospho-paxillin\(^{31}\) was markedly altered in TOT sections. Patchy labeling observed in nontransgenic control sections (Fig. 4B) lacked the pronounced striations of phospho-paxillin\(^{31}\) reactivity apparent in TOT sections (Fig. 4C). These myofibril-like patterns of anti-phospho-paxillin\(^{31}\) showed varying degrees of disorganization, probably related to the degeneration of myofibril structure concurrent with development of dilatation in TOT cardiomyocytes (10). Accumulation of another protein belonging to focal adhesion complexes, vinculin, was also found with increased expression in TOT lysates compared with nontransgenic samples (data not shown). Thus, phospho-paxillin\(^{31}\) reactivity is increased in TOT hearts and shows altered distribution, suggesting changes in focal adhesion complex signaling in these diluted hearts.

**Tmod Overexpression in Cultured Cardiomyocytes Reproduces Some, but Not All, of the Signaling Phenotype Observed in TOT Hearts**—Neonatal rat cardiomyocytes were infected with recombinant adenoviruses to determine whether overexpression of Tmod can mimic the observed *in vivo* phenotype in terms of content and activation of pyk2/RAFTK, FAK, paxillin, and vinculin. Cultures were infected with adenoviruses expressing Tmod or \(\beta\)-gal as a control and then processed for immunoblot analysis or confocal microscopy. Tmod expression in the soluble fraction was increased 3.9-fold following adenoviral expression as measured by immunoblot analysis (data not shown), demonstrating efficient expression of the virally encoded protein. In contrast to myocardial samples, total content and activation level of pyk2/RAFTK or FAK were unaffected by Tmod expression relative to either \(\beta\)-gal-infected cultures or whole cell extracts from non-infected cardiomyocytes (data not shown). However, immunoblot analyses showed increased immunoreactivity for phospho-paxillin\(^{31}\) and vinculin compared with \(\beta\)-gal or non-infected cells (Fig. 5A). As was found *in vivo*, increased changes were not correlated to elevation of total paxillin content (data not shown). Quantitation of these increases by immunoblot analysis demonstrated a 3.1-fold elevation of phospho-paxillin\(^{31}\) and a 1.7-fold increase in vinculin content resulting from Tmod overexpression relative to \(\beta\)-gal infection (Fig. 5A). The distribution of the focal adhesion component proteins phospho-paxillin\(^{31}\) and vinculin was also determined by confocal microscopy of cultured cardiomyocytes (Fig. 5B). Increased staining for both phospho-paxillin\(^{31}\) and vinculin was evident after Tmod expression (Fig. 5B, bottom row), as would be expected based upon immunoblot results (Fig. 5A). Immunolocalization showed each protein was coincident with \(\alpha\)-actinin, which labeled myofibril Z-discs with similar intensity in cultures that were uninfected (Fig. 5B, top row), infected with virus expressing \(\beta\)-galactosidase (middle row), or infected with virus expressing Tmod (bottom row). These results demonstrate that both phospho-paxillin\(^{31}\) and vinculin accumulate in the region of the Z-disc where structures analogous to focal adhesions, called costameres, are concentrated. Collectively, these *in vitro* experiments demonstrate that Tmod overexpression induces certain phenotypic changes similar to those observed in TOT heart in isolated neonatal cardiomyocytes, at least related to two proteins associated with focal adhesion complex organization. Moreover, these results indicate that accumulation of Tmod alone does not lead to a generalized activation of kinase pathways associated with adhesion remodeling, suggesting that the activation observed in TOT myocardial samples is related to cardiac dilation and heart failure. This discrepancy between the *in vivo* and *in vitro* activation of pyk2/RAFTK following Tmod overexpression is likely caused by elevated calcium levels in the TOT mouse cardiomyocytes (9).

**Elevation of Intracellular Calcium Activates pyk2/RAFTK in Cultured Cardiomyocytes**—The TOT phenotype is associated with elevation of intracellular calcium that could account for the activation of the calcium-dependent pyk2/RAFTK kinase. To test this hypothesis *in vitro*, cultured cardiomyocytes were treated with calcium ionophore for 25 min and phospho-pyk\(^{402}\) levels were assessed by immunoblot. Increased phospho-pyk\(^{402}\) level was found in those cardiomyocytes treated with 0.5 \(\mu\)M ionomycin (Fig. 6). This calcium-mediated activation of pyk2/RAFTK was not affected by accumulation of adenosvirally overexpressed Tmod. These results indicate that pyk2/RAFTK activation can be mediated by elevation of intracellular calcium concentration in cardiomyocytes and also demonstrate that Tmod accumulation alone does not promote calcium-mediated signal transduction.

**pyk2/RAFTK Overexpression in Cultured Cardiomyocytes Increases Paxillin Phosphorylation and Induces Myofibrillar Remodeling**—Because pyk2/RAFTK-mediated signaling is activated in TOT hearts (Fig. 1), the effect of increased pyk2/RAFTK signaling in cardiomyocytes was examined using recombinant adenosviruses expressing either pyk2/RAFTK-WT or a phosphorylation-deficient mutant (pyk2/RAFTK-Tyr\(^{402}\)). Immunoblot analyses demonstrate that a modest level of pyk2/RAFTK immunoreactivity found in uninfected or \(\beta\)-gal-expressing cardiomyocytes is dramatically increased by infection with adenosviruses encoding either pyk2/RAFTK-WT or pyk2/...
leads to increased levels of both phospho-pyk2/RAFTK Tyr402 and 45208 of total cell extract, and ionophore treatment was performed for 25 min. (control) cell lysate and lysate from cultures infected with either control examined for accumulation of phospho-pyk2/RAFTK 402. Uninfected of cultured cardiomyocyte lysates treated with ionophore and then by elevation of intracellular calcium. the appropriate control sample. The experiment was repeated twice with comparable results both times. All lanes were loaded with 100 μg of total cell extract, and ionophore treatment was performed for 25 min.

RAFTK-Tyr402 (Fig. 7). Infection with the pyk2/RAFTK-WT leads to increased levels of both phospho-pyk2/RAFTK Tyr402 and phospho-paxillin31, whereas accumulation of pyk2/RAFTK-Tyr402 has no effect on phosphorylation state of either protein. Interestingly, overexpression of pyk2/RAFTK-WT diminished the level of total paxillin present in the lysates without affecting total FAK expression, suggesting that pyk2/RAFTK-mediated phosphorylation could play a role in regulation of paxillin content and/or distribution. Accumulation of pyk2/RAFTK-WT in cultured cardiomyocytes leads to marked loss of myofibril organization from peripheral regions of the cell (Fig. 8), although sarcomeric structures are readily apparent in the cell center. Collectively, these results indicate that adenosvirally mediated expression of pyk2/RAFTK-WT protein leads to decreased paxillin level but increased paxillin phosphorylation, which may be involved in the dramatic myofibril remodeling phenotype induced by pyk2/RAFTK-WT in cultured cells.

**DISCUSSION**

The membrane cytoskeleton is an integral part of cellular architecture connecting transmission of force between the cell interior and the extracellular environment. Structural reorganization is requisite to the cardiac remodeling process (7, 8, 27), but the impact of hypertrophy or dilation upon cardiomyocyte cytoskeletal organization remains largely unknown. This study establishes a correlation between activation of signaling leading to focal adhesion remodeling and cardiomyopathic changes in the TOT mouse model of dilation.

pyk2/RAFTK is activated by a plethora of stimuli including hormones, growth factors, chemokines, cytokines, stress-related signals, adherence, and pharmacological agents that elevate intracellular calcium or activate protein kinase C (11, 28–30). pyk2/RAFTK signaling has been extensively characterized in non-muscle cells (18, 31, 32) where postulated effects include coordinate regulation of cytoskeletal protein phosphorylation in combination with FAK (11, 34). pyk2/RAFTK signaling related to cardiovascular regulation has been previously examined in the context of vascular smooth muscle, cardiac fibroblasts, and cardiomyocytes. pyk2/RAFTK expression is developmentally regulated in heart (14), although it is more abundant in vascular smooth muscle cells, where activation is dependent of angiotensin II (35). In cardiac fibroblasts, where pyk2/RAFTK is highly expressed, angiotensin II mediates regulation in a Ca2+/calmodulin-sensitive manner (36). Our results extend this list with the novel observation that cardiomyopathic remodeling is associated with activation of pyk2/RAFTK signaling (Fig. 1).

pyk2/RAFTK and FAK are highly homologous tyrosine kinases that coordinately regulate actin cytoskeleton organization. Examination of cross-talk demonstrated the ability of pyk2/RAFTK to phosphorylate FAK (11), and both molecules are substrates for Src kinase (11, 23). Although pyk2/RAFTK and FAK exhibit functional redundancy under certain experimental conditions (11), variations in the C-terminal domain of the two molecules may confer distinct functional activities (37). Differences do exist, because activation of FAK occurs predominantly via integrin engagement (38, 39), pyk2/RAFTK cannot fully compensate for loss FAK (21), and functional roles for pyk2/RAFTK and FAK are markedly distinct under certain conditions (40, 41). Participation of FAK in cardiovascular signaling occurs in cardiomyocytes responding to hypoxia (42), pulsatile stretch (43, 44), vascular endothelial growth factor (45), and hypertrophy in vivo (46, 47) and in vitro (48–51).
These reports linking FAK with alteration of cardiomyocyte structure or adhesion are consistent with FAK activation occurring in TOT cardiomyocytes undergoing cytoskeletal remodeling (Fig. 2). Colocalization of FAK with paxillin in TOT heart sections (Fig. 2) places FAK in proximity to focal adhesion complexes where paxillin is concentrated. FAK is known to interact with and phosphorylate paxillin (22, 52, 53), and paxillin acts as a multifunctional adaptor protein to bind regulatory proteins and transduce signals related to focal adhesion structural organization (52). Thus, FAK is likely to act as a regulator of adhesion via paxillin phosphorylation in the remodeling TOT heart.

Focal adhesions are macromolecular structures enriched for many molecules that serve roles in cytoskeletal architecture, signal transduction, or both (54). Cytoskeletal and sarcomeric actin filaments terminate in adhesions and are anchored by interaction with various actin-binding proteins concentrated at adhesions such as vinculin and α-actinin. Vinculin accumulates both in TOT mice (Fig. 4) or cardiomyocytes overexpressing Tmod (Fig. 5), which correlates with a previous demonstration of increased vinculin resulting from impaired cardiomyocyte contractility (55). Adhesion complexes and their constituent proteins have been extensively studied in vitro, but relatively little is known about their in vivo counterparts. For cardiomyocytes, the corresponding structure is probably the costamere: a “rib-like” structure overlying the Z-disc perimeter that acts as the site of force transmission to the substratum (56). Costameric localization would account for the striated appearance of phospho-paxillin31 labeling in TOT heart sections (Fig. 4). Increased phospho-paxillin31 labeling is associated with altering adhesion, cytoskeletal remodeling, and growth control (54), suggesting adhesion remodeling is underway in TOT (Fig. 4) hearts as well as cardiomyocytes undergoing myofibril degeneration (Fig. 5). The presence of the faster mobility 38-kDa phosphoprotein postulated to be a fragment of paxillin (Fig. 4) correlates with a recent report of a caspase-mediated early cleavage site at residue Asp301 (25). Apoptotic cell death is present in the TOT model of cardiomyopathy and contributes to pathogenesis (9), although the specific role of caspases in pathogenesis is unknown. The additional larger phosphoprotein in Fig. 4 that parallels increased immunoreactivity of the presumptive paxillin fragment at 38 kDa could be another paxillin fragment of ~55 kDa reported to be produced by the action of calpain I, which contributes to dissolution of focal adhesion complexes (26). FAK activation is consistent with phosphorylation of paxillin at tyrosine residue 31, which is a predominant target of FAK (57). Because focal adhesion complexes are also likely to be important for maintenance of contractility, the remodeling and loss of these structures may contribute to impaired cardiac function in TOT hearts (10).

Elevation of intracellular calcium level in TOT cardiomyocytes (6, 9) triggers pyk2/RAFTK activation, as suggested by experiments in cultured cardiomyocytes. Tmod accumulation leads to loss of myofibril organization and concomitant inhibition of contractility (10), consistent with decreased level of phospho-pyk2/RAFTK402 immunoreactivity (Fig. 6). Importantly, this result indicates that Tmod accumulation alone cannot account for the activation of pyk2/RAFTK signaling observed in the TOT heart and that additional required stimuli are present in vivo. Thus, Tmod expression alone does not artificially activate calcium-dependent signaling in cultured cells but instead diminished contractility and pyk2/RAFTK activity, as opposed to enhanced contractility (and concomitant enhancement of calcium dynamics) that stimulated pyk2/RAFTK activation (14). The increase of phospho-pyk2/RAFTK402 level observed following exposure to calcium iono-
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Ated with hypertrophy and dilation (3, 4). Multiplex signaling occurring in TOT cardiomyopathy may have relevance for end stage human heart failure characterized by activation of numerous reactive pathways (33), as well as elevation of intracellular calcium levels leading to activation of calcium-dependent signaling (1, 2). Thus, TOTs are a useful paradigm for dissecting the relative contributions of these signaling mechanisms in the transition from adaptation to compensation. Identification of pyk2/RAFTK-mediated signaling in cardiomyocytes sets the stage for future studies to encompass a wider range of adhesion-associated signaling molecules, as well as determine the contribution of adhesion remodeling in the pathogenesis of cardiomyopathy.

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REFERENCES

1. Beuckelmann, D. J., Naber, M., and Erdmann, E. (1992) Circulation 85, 1046–1055
2. Richard, S., Ledez, F., Lemaire, S., Piot, C., and Nargeot, J. (1998) Cardiovasc. Res. 37, 300–311
3. Bowman, J. C., Steinberg, S. F., Jiang, T., Geenen, D. L., Fishman, G. L., and Buttrick, P. M. (1997) J. Clin. Invest. 100, 2169–2195
4. Wakasakai, H., Koya, D., Schoen, F. J., Jirousek, M. R., Ways, D. K., Hie, B. D., Walsh, R. A., and King, G. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8202–8207
5. Molkentin, J. D., Lu, J. R., Antos, C. L., Marham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. (1998) J. Cell. Biol. 141, 1711–1718
6. Sussman, M. A., Welch, S., Gude, N., Khoury, P. R., Daniels, S. R., Durr, J. R., and Groopman, J. E. (1997) J. Biol. Chem. 272, 514–521
7. Sussman, M. A., Lim, H. W., Gude, N., Taigen, T., Olson, E. N., Robbins, J., Pasztor, L., White, R., Groopman, J. E. (1997) J. Biol. Chem. 272, 10458–10465
8. Sussman, M. A., Welch, S., Walker, A., Klevitsky, R., Hewett, T., E., Price, R. B., Lim, H. W., and Molkentin, J. D. (1999) Am. J. Pathol. 155, 2101–2113
9. Welch, S., Plank, D., Witt, S., Glascock, B., Chimenti, S., Andreoli, A. M., Limana, F., Leri, A., Kajstura, J., Anversa, P., and Sussman, M. A. (2002) Circ. Res. 90, 649–656
10. Sussman, M. A., Welch, S., Cambon, N., Klevisky, R., Hewett, T., Witt, S. A., and Kimball, T. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10192–10197
11. Li, X., Dy, R. C., Cance, W. G., Graves, L. M., and Earp, H. S. (1999) J. Biol. Chem. 274, 10140–10144
12. Pandey, P., Avraham, S., Kumar, S., Nakazawa, A., Place, A., Ghanem, L., Sussman, L., Srivastava, A., Srivastava, A. K., and Groopman, J. E. (1999) J. Biol. Chem. 274, 10140–10144
13. Brines, A. E., Harding, T., Dilhoretz, P. A., Her, Y. L., Xie, H., Hunter, D., Herman, B., Earp, H. S., and Graves, L. M. (1998) J. Biol. Chem. 273, 1714–1718
14. Bayer, A. L., Ferguson, A. G., Luchetti, P., and Samarel, A. M. (2001) J. Mol. Cell. Cardiol. 33, 1017–1030
15. Bayer, A. L., Heidkamp, M. C., Patel, N., Porter, M. J., Engman, S. J., and Samarel, A. M. (2002) Am. J. Physiol. 283, H1695–H1706
16. Turner, C. E., and Miller, J. T. (1994) J. Cell Sci. 107, 1583–1591
17. Clerk, A., Bogoyevitch, M. A., Andersson, M. B., and Sudgen, P. H. (1994) J. Biol. Chem. 269, 28248–28257
18. Avraham, S., London, R., Fu, Y., Otta, S., Hiregordara, D., Li, J., Jiang, S., Pasztor, L., White, R., Groopman, J., and Avraham, H. (1995) J. Biol. Chem. 270, 27742–27751
19. Ganja, R. K., Hatch, W. A., Avraham, H., Ona, M. A., Drucker, B., Avraham, S., and Groopman, J. E. (1997) J. Exp. Med. 185, 1055–1063
20. Kozak, K., Kazemzadeh, P., Park, S. Y., Fu, Y., Avraham, S., and Avraham, H. (2001) Br. J. Haematol. 114, 134–140
21. Sieg, D. J., Ilic, D., Jones, K. C., Dameky, C. H., Hunter, T., and Schlaepfer, D. D. (1998) EMBO J. 17, 5933–5947
22. Tchekhova, R., Sato, T., D’Avrero, N., and Morimoto, C. (1995) J. Exp. Med. 182, 1089–1100
23. Calabab, M. B., Zhang, X., Polte, T. R., and Hanks, S. K. (1996) Biochem. Biophys. Res. Commun. 228, 602–608
24. Bellis, S. L., Miller, J. T., and Turner, C. E. (1995) J. Biol. Chem. 270, 17437–17441
25. Chay, K.-O., Park, S. K., and Mushinski, J. F. (2002) J. Biol. Chem. 277, 14521–14529
26. Carragher, N. O., Levkau, B., Ross, R., and Raines, E. W. (1999) J. Cell Biol. 143, 619–629
27. Francis, G. S. (1998) Circ. Opin. Cardio. 13, 156–161
28. Della Rocca, G. J., Maudsley, S., Daaka, Y., Leffkowitz, R. J., and Luttrell, L. M. (1995) J. Biol. Chem. 270, 3773–3785
29. Liu, Z. Y., Ganju, R. K., Wang, J. F., Ona, M. A., Hatch, W. C., Zheng, T., Avraham, S., Gill, P., and Groopman, J. E. (1997) J. Clin. Invest. 99, 1804–1809
30. Mikkopadhyay, D., Nagy, J. A., Manseau, E. J., and Dvorak, H. F. (1998) Cancer Res. 58, 1278–1284
31. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Howman, G. D., Rudy, H. E. and Schlesinger, J. (1995) Nature 376, 737–745
32. Sasahara, S., Nagura, K., Ishino, M., Tobihisa, H., Katani, K., Sasaki, T. (1995) J. Biol. Chem. 270, 21206–21219
33. Buonomo, I., Walsh, R. A., Song, G., Estridge, T., Sandusky, G. E., Fouts, R. L., Mintez, K., Pickard, T., Roden, B., Ristroph, M. R., Sabbah, H. N., Mizrahi, J. L., Gromo, K. G., and Vlahos, C. J. (1999) Circulation 99, 2583–2591
34. Du, Q.-S., Ren, X.-R., Yue, X., Wang, Y., Mei, L., and Xiong, W.-C. (2001) J. Cell Sci. 114, 2977–2987
35. Rocic, P., Govindarajan, G., Sabri, A., and Lucehesi, P. A. (2001) Am. J. Physiol. 280, C950–C956
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