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Cardiomyocyte Apoptosis Triggered by RAFTK/pyk2 via Src Kinase Is Antagonized by Paxillin*

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Altered cellular adhesion and apoptotic signaling in cardiac remodeling requires coordinated regulation of multiple constituent proteins that comprise cytoskeletal focal adhesions. One such protein activated by cardiac remodeling is related adhesion focal tyrosine kinase (RAFTK, also known as pyk2). Adenoviral-mediated expression of RAFTK in neonatal rat cardiomyocytes involves concurrent increases in phosphorylation of Src, c-Jun N-terminal kinase, and p38 leading to characteristic apoptotic changes including cleavage of poly(ADP-ribose) polymerase, caspase-3 activation, and increased DNA laddering. DNA laddering was decreased by mutation of the Tyr^{402} Src-binding site in RAFTK, suggesting a central role for Src activity in apoptotic cell death that was confirmed by adenoviral-mediated Src expression. Multiple apoptotic signaling cascades are recruited by RAFTK as demonstrated by prevention of apoptosis using caspase-3 inhibitor IV (caspase-3 specific inhibitor), PP2 (Src-specific kinase inhibitor), or Csk (cellular negative regulator for Src), as well as dominant negative constructs for p38β or MKP-1. These RAFTK-mediated phenotypic characteristics are prevented by concurrent expression of wild-type or a phosphorylation-deficient paxillin mutated at Tyr^{31} and Tyr^{118}. Wild-type or mutant paxillin protein accumulation in the cytoplasm has no overt effect upon cell structure, but paxillin accumulation prevents losses of myofibril organization as well as focal adhesion kinase, vinculin, and paxillin protein levels mediated by RAFTK. Apoptotic signaling cascade inhibition by paxillin indicates interruption of signaling proximal to but downstream of RAFTK activity. Chronic RAFTK activation in cardiac remodeling may represent a maladaptive reactive response that can be modulated by paxillin, opening up novel possibilities for inhibition of cardiomyocyte apoptosis and structural degeneration in heart failure.

Heart failure is characterized by cellular remodeling and apoptosis of cardiomyocytes (1–3). The precipitating stimulus of chronically impaired calcium handling promotes maladaptive remodeling via activation of calcium-dependent signaling cascades (4, 5) with chronic elevation of calcium influx leading to hypertrophy and heart failure in transgenic mice (6). Earlier work from our group demonstrated activation of related adhesion focal tyrosine kinase (RAFTK, also known as pyk2) signaling in cultured cardiomyocytes treated with ionomycin (7) as well as a murine model of dilated cardiomyopathy exhibiting chronic elevation of intracellular calcium levels (8, 9). Concurrent with RAFTK/pyk2 activation, heart samples from cardiomyopathic mice showed enhanced paxillin phosphorylation (7). Thus, paxillin was implicated in the RAFTK/pyk2 signaling cascade leading to heart failure as a target substrate of RAFTK/pyk2-mediated phosphorylation.

RAFTK/pyk2 signaling has been extensively characterized in nonmuscle cells where postulated effects include coordinate regulation of cytoskeletal protein phosphorylation in combination with FAK[1] (10). *RAFTK/pyk2* is activated by a plethora of stimuli including hormones, growth factors, chemokines, cytokines, stress-related signals, and cellular adherence that elevate intracellular calcium or activate protein kinase C (11–14). Activated RAFTK/pyk2 binds and activates Src kinase through the Src homology 2 domain at tyrosine 402 (Tyr^{402}) of RAFTK/pyk2 (15). Cumulative published studies suggest that the involvement of the RAFTK/pyk2-Src signaling axis in cell survival or death is context dependent. Apoptotic signaling has been linked to RAFTK/pyk2 wild-type (WT) expression in thymocytes, whereas kinase inactive RAFTK/pyk2 or treatment with the Src kinase inhibitor PP1 blocked apoptosis (16). In cardiomyocytes, inhibition of Src or caspase activity delays apoptotic signaling and detachment (17), but a recent study implicated phosphorylation of both RAFTK/pyk2 and Src in endothelin-1-mediated cardiomyocyte survival (18). We previously observed that Src activation *in vivo* can be detrimental (19). Csk-expressing adenovirus, which inhibits Src by phosphorylation of Tyr^{527}, has been shown to block apoptotic signaling (20, 21). Csk also influences paxillin phosphorylation (22–24), although the cellular impact of this regulation remains to be elucidated. Similar effects were observed in breast cancer cells where Csk homologous kinase associates with and inhibits phosphorylation of RAFTK/pyk2, FAK, and paxillin (25).

RAFTK/pyk2 signaling related to cardiovascular regulation has been examined previously in the context of vascular smooth muscle, cardiac fibroblasts, and cardiomyocytes.

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[1] The abbreviations used are: FAK, focal adhesion kinase; WT, wild-type; JNK, c-Jun NH_2-terminal kinase; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase.

RAFTK/pyk2 participates in attachment to extracellular matrix, cytoskeletal rearrangement, and apoptosis (16, 26, 27). RAFTK/pyk2 expression is developmentally regulated in the heart and abundant in vascular smooth muscle cells where activation of ERK1/2 mitogen-activated protein kinases and phosphatidylinositol 3-kinase is influenced by RAFTK/pyk2. RAFTK/pyk2 concentration in cardiomyocytes is much greater in neonates than adults and is highly dependent on calcium transients and contractility (26). Consistent with our findings in the pathologically altered myocardium (7), increased expression and phosphorylation of RAFTK/pyk2 were noted following left ventricular hypertrophy induced by pressure overload (28). We found overexpression of WT RAFTK/pyk2 caused dramatic changes in myofibril organization consistent with a role in reorganization of focal adhesions and cytoskeleton and demonstrated that RAFTK/pyk2 is activated in dilated cardiomyopathy (7). Chronic elevation of intracellular calcium in cardiomyocytes of failing hearts (8, 9) is presumably responsible for RAFTK/pyk2 activation, which then leads to redistribution and altered phosphorylation of paxillin (7).

Paxillin, a constituent member of focal adhesions, serves as an adaptor protein that associates with signaling and cytoskeletal molecules involved in the regulation of focal adhesion dynamics such as RAFTK/pyk2, FAK, c-Src, Csk, protein-tyrosine-phosphatase-PEST, talin, and vinculin (29, 30). Evidence of cleaved paxillin in samples from murine cardiomyopathic hearts (7) prompted further investigation into the relationship between RAFTK/pyk2 and paxillin, which was found in this study to involve activation of Src kinase and cell death.

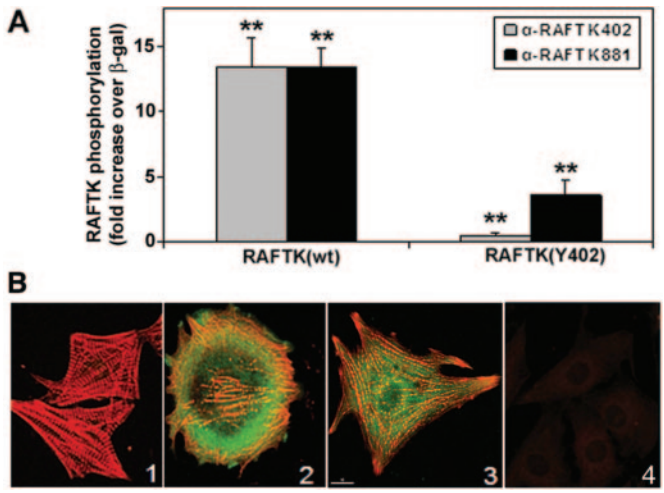

FIG. 1. **Overexpression of RAFTK/pyk2 enhances kinase activity and induces remodeling.** *A*, quantitation of immunoblots for phosphorylation on residues Tyr^{402} and Tyr^{881} of RAFTK/pyk2 in cultured cardiomyocyte lysates. RAFTK/pyk2 (WT) shows dramatically enhanced phosphorylation relative to a RAFTK/pyk2 (Tyr^{402}) mutant construct following adenovirally mediated overexpression. *B*, confocal micrographs showing cells infected with adenovirus expressing β-gal (*1*) RAFTK/pyk2 (WT) (*2*), RAFTK/pyk2 (Tyr^{402}) (*3*), or uninfected cardiomyocytes showing endogenous RAFTK/pyk2 (*4*). RAFTK/pyk2 immunoreactivity (*green*) is not observed from β-gal controls. Myofibril-associated actin filament organization revealed by labeling with phalloidin (*red*) is markedly diminished by RAFTK/pyk2 (WT) expression, particularly in peripheral regions of the cell. In comparison, RAFTK/pyk2 (Tyr^{402}) accumulation does not markedly perturb myofibril organization.

EXPERIMENTAL PROCEDURES

Antibodies and Chemical Reagents—Phosphorylated sites on signaling proteins were detected using antibodies to RAFTK/pyk2 ($Tyr(P)^{402}$ and $Tyr(P)^{881}$), Src ($Tyr(P)^{416}$), and paxillin ($Tyr(P)^{31}$ and $Tyr(P)^{118}$) from BIOSOURCE-QCB, (Camarillo CA), and phospho-c-Jun N-terminal protein kinase (JNK) and phospho-p38 (all from BIOSOURCE). Membranes were reprobed with anti-RAFTK/pyk2 and anti-paxillin monoclonal antibodies (both from Transduction Laboratories, Lexington KY), anti-phosphotyrosine, anti-JNK, anti-p38, and anti-Src antibodies (all from Cell Signaling Technology, Beverly MA). Anti-vinculin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-FAK polyclonal antibody was purchased from Upstate Laboratories (Waltham, MA). For immunoblot studies, alkaline phosphatase-conjugated antibodies directed against goat, rabbit, and mouse IgG were used (all from Santa Cruz Biotechnology). For confocal studies, primary antibodies were detected by fluorescently tagged (fluorescein isothiocyanate) secondary IgG antibodies directed against the appropriate primary host species as well as phalloidin labeled with Texas Red or Cy5 fluorophores (all from Molecular Probes, Eugene, OR). Ribonuclease A (Sigma), cleaved caspase-3 polyclonal antibody (Cell Signaling), proteinase K (Invitrogen) and anti-cleaved-anti-PARP site (214/215) polyclonal antibody (BIOSOURCE-QCB) were used for experiments related to apoptotic signaling. Caspase-3 inhibitor IV Ac-Asp-Met-Gln-Asp-CHO (Ac-DMQD-CHO) and PP2 (both from Calbiochem) and Z-Asp-Glu-Val-Asp-chloromethylketone (Z-DEVD-cmk, Bachem, Torrance CA) were used to inhibit apoptotic signaling cascades.

Neonatal Rat Ventricular Myocytes Isolation—Neonatal rat ventricular myocytes were isolated from hearts of 2–3-day-old Sprague-Dawley rats by multiple collagenase type I (Worthington) and pancreatine (Sigma) digestions as described previously (31). Myocytes were preplated for 2 h in M-199 plus 15% fetal bovine serum to reduce nonmyocyte contamination and plated at various densities either on plastic chamber slides pretreated with laminin (Sigma) or 1% gelatin-coated dishes and left in a 5% CO_2 incubator for 18 h. The next day, myocytes were washed and refed with serum-free M-199 culture medium.

Adenoviruses Constructs and Infections—Rat *RAFTK/pyk2*, paxillin cDNA fragments encoding both WT and phosphorylation-deficient mutants (at Tyr^{402} for *RAFTK/pyk2*, Tyr^{31}-Tyr^{118} for paxillin), and Src kinase cDNAs (Upstate Biotechnology) were used to create replication-defective recombinant adenoviruses with the Ad Easy system (Microbix Biosystem, Inc., Ontario, Canada) as directed by the manufacturer. Dominant negative adenoviruses for MKP-1 and P38β were kindly provided by Dr. J. Molkentin (Children's Hospital Research Foundation, Cincinnati, OH). Csk adenoviruses were graciously provided by Dr. S. Tanaka (Faculty of Medicine, University of Tokyo, Tokyo, Japan). Subconfluent cardiomyocyte cultures maintained in serum-free conditions for 24 h were infected with recombinant adenoviruses, including β-galactosidase (β-gal)-expressing adenovirus as a control for 2 h at a multiplicity of infection of 60:1, and then medium was aspirated and replaced with maintenance medium. Lysates for biochemical studies were prepared ~24 h after infection. For co-infection experiments, initial infections were performed with one adenovirus and allowed to proceed for 24 h to allow for protein accumulation prior to secondary adenoviral infection.

Inductors and Inhibitors of Apoptosis—Cardiomyocytes were treated with staurosporine (1 μM, for 12 h) to induce apoptosis, whereas inhibition of apoptosis was mediated by 2-h treatment with either 10–100 μM concentrations of PP2, caspase-3 inhibitor IV (Ac-DMQD-CHO), or Z-DEVD-cmk. All infections were all performed 24 h after respective treatments.

Immunoblotting—Lysates from neonatal rat ventricular myocytes cultures were washed twice with phosphate-buffered saline and lysed on ice in lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EGTA) containing a mixture of protease (100 μM sodium orthovanadate and NaF, 10 μM sodium pyrophosphate, 1 mM dithiothreitol and 10 μg/ml each pepstatin, leupeptin, aprotinin, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, and L-1-tosylamido-2-phenylethyl chloromethyl ketone) and phosphatase (10 nM each cypermethrin and okadaic acid and 100 μM phenylasine oxide) inhibitors. Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes that were probed with various primary antibodies as indicated and detected by enhanced chemifluorescence according to manufacturer protocols (Amersham Biosciences). The membranes were subjected to immunoblot analysis with antibodies to phospho-Src-529, phospho-JNK, and/or phospho-p38-MAPK. Membranes were stripped and reprobed with either anti-pan-Src-, JNK-, or p38-MAPK antibodies. Blots were quantified by laser scanning densitometry. Results were expressed as the ratio phosphorylated protein kinase form to total protein kinase level. All blots were controlled for equal loading using glyceraldehyde-3-phosphate dehydrogenase protein level determination as a standard. For reprobing, membranes were stripped overnight in stripping solution (30% sulfosalicylic acid, 30% trichloroacetic acid, 0.5% Ponceau S), washed, blocked, and reprobed.

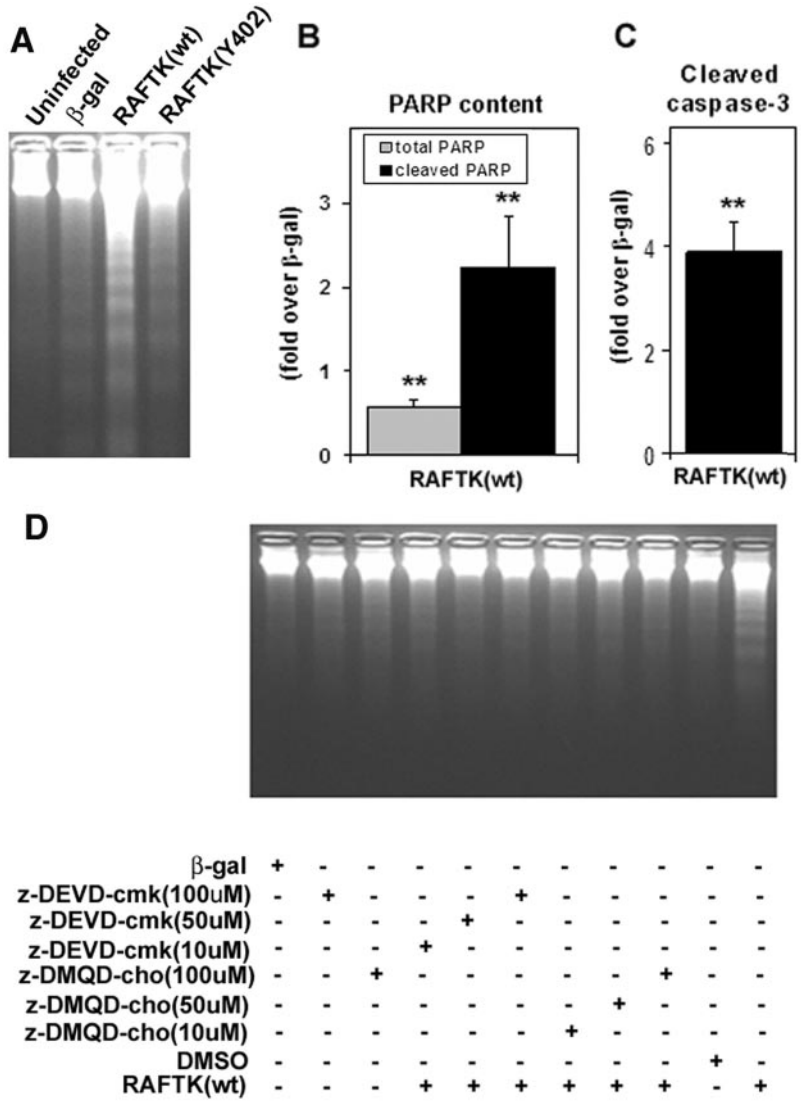

FIG. 2. **RAFTK/pyk2 promotes apoptotic signaling.** Apoptotic signaling in cultured cardiomyocytes evaluated by DNA laddering (*A*), PARP cleavage (*B*), and caspase-3 cleavage (*C*). Cells were infected with adenoviruses expressing RAFTK/pyk2 (WT) or RAFTK/pyk2 (Tyr^{402}) mutant construct as indicated and processed for analyses 24 h later. *A*, DNA fragmentation is enhanced by infection with RAFTK/pyk2 (WT) relative to cultures infected with RAFTK/pyk2 (Tyr^{402}) mutant construct. Cellular DNA (8–10 μg) was separated using 2% agarose gels and revealed by ethidium bromide staining. *B*, total and cleaved-PARP signals show significant increases in PARP cleavage with RAFTK/pyk2 (WT). *C*, cleaved caspase-3 level is significantly increased by RAFTK/pyk2 (WT). Signal intensities were corrected for effects of adenoviral infection by standardization to β-gal controls, followed by correction for minor loading variations by normalization to GAPDH signal. **, $p < 0.01$ *versus* β-gal infection. *D*, inhibition of caspase-3 cleavage by 2-h pretreatment with Z-DEVD-cmk or Z-DMQD-CHO (caspase-3 inhibitor IV; 10–100 μM) prevents DNA laddering mediated by subsequent challenge with RAFTK/pyk2.

Immunostaining—Cardiomyocytes cultured in plastic chamber slides pretreated with laminin were fixed in 4% paraformaldehyde in phosphate-buffered saline containing 0.2% Triton X-100 and blocked with 10% horse serum (Invitrogen) for 1 h at room temperature. Primary antibodies were left overnight followed by secondary antibody incubation for 2 h. Slides were washed, mounted, and evaluated by confocal microscopy. Cardiomyocyes were readily identified by labeling with Texas Red-phalloidin to visualize myofibrils of neonatal rat ventricular myocytes.

DNA Fragmentation—Cardiomyocytes were treated with 1 ml of lysis buffer (0.8 mM EDTA (pH 8.0), 8 mM Tris-HCl (TE, pH 8.0) and 4% SDS). DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. Resulting DNA was incubated with proteinase K (50 μg/ml) for 2 h at 50 °C to facilitate protein disruption. After a second extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), DNA was precipitated from the upper aqueous phase with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol at −20 °C overnight. The next day, DNA was centrifuged, washed, dried, and resuspended in TE buffer followed by a 60-min incubation with ribonuclease A (2 mg/ml) at 37 °C. Samples were re-extracted, and DNA was precipitated as described above. Finally, pellets were resuspended in TE buffer, and DNA concentrations were quantified from the absorbance at 260 nm. 8–10 μg of DNA samples were analyzed by electrophoresis on 2% agarose gels and visualized by staining with a solution containing 0.2 μg/ml of ethidium bromide.

Expression of Results and Statistical Analysis—Data are represented as means ± S.E. of the number of independent experiments indicated (*n*) or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by analysis of variance, and comparisons between groups were performed using a protected Tukey's *t* test. Values of $p < 0.01$ and $p < 0.05$ were set as the limit of statistical significance.

RESULTS

Adenoviral-mediated Expression of RAFTK/pyk2 Enhances Kinase Activity and Induces Alteration of Cardiomyocyte Structure—Neonatal rat cardiomyocytes were infected with recombinant adenoviral vectors expressing RAFTK/pyk2 (WT) or a mutated construct with impaired Src-binding activity (Tyr^{402}). Infected cardiomyocytes showed accumulation of RAFTK/pyk2 (WT or Tyr^{402} mutant) in the cytoplasm and increases of 4.8- and 5.2-fold, respectively over β-gal expression. Overexpression of RAFTK/pyk2 (WT) increased the level of detectable phosphorylation at either residue Tyr^{402} or Tyr^{881} over 13-fold relative to control cultures infected with adenovirus expressing β-gal, consistent with activation (Fig. 1*A*). In contrast, the detectable phosphorylation level of the Tyr^{402} residue following expression of the mutant RAFTK/pyk2 (Tyr^{402}) was reduced 0.5-fold relative to β-gal controls with a moderate rise in Tyr^{881} phosphorylation of 3.6-fold (Fig. 1*A*). RAFTK/pyk2 (WT) overexpression leads to marked cardiomyocyte remodeling and loss of myofibril organization mainly in peripheral regions whereas intact myofibrillar structures were maintained in the cell center (Fig. 1*B*, *2*). In comparison, RAFTK/pyk2 (Tyr^{402}) overexpression did not

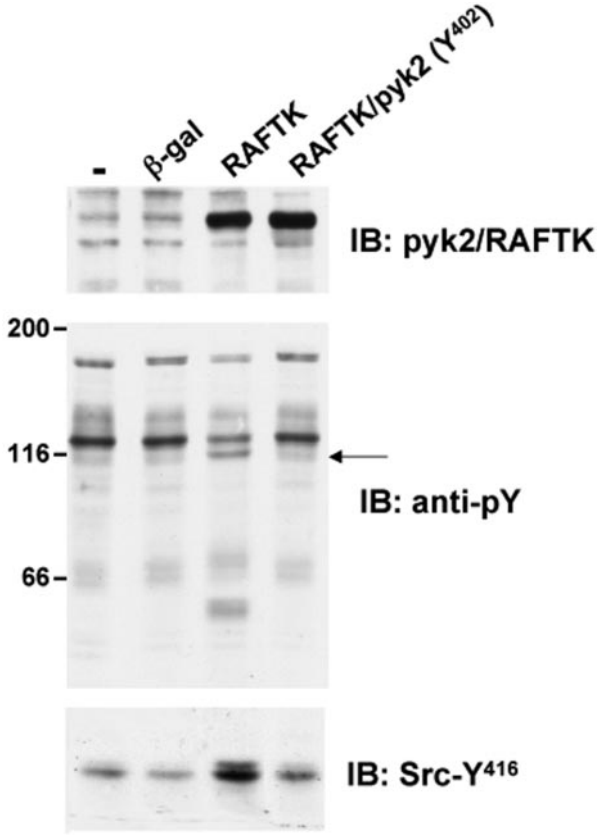

FIG. 3. **Increased phosphorylation of Src by RAFTK/pyk2.** Immunoblots of cardiomyocyte cultures that are uninfected (−) or infected with adenoviruses expressing β-gal, RAFTK/pyk2, or RAFTK/pyk2 (Tyr^{402}) mutant construct. *Top panel* of blots shows labeling with RAFTK/pyk2 antibody, the *middle panel* shows labeling with phosphotyrosine antibody, and the *bottom panel* shows labeling with phospho-Src (Tyr^{416}) antibody. Phosphotyrosine labeling for RAFTK/pyk2 is indicated by the *arrow* for the band appearing following expression of RAFTK (WT), consistent with mobility as determined by molecular weight markers indicated on the *left* of the blot (116 kDa, *middle panel*, at *arrow*). The upper band of immunoreactivity present in all lanes is endogenous labeling of another phosphotyrosine protein, possibly focal adhesion kinase (see "Results").

promote loss of myofibril organization, and peripheral attachments of myofibrils remained intact (Fig. 1*B*, *3*).

RAFTK/pyk2 Expression Induces Apoptotic Signaling in Cardiomyocytes—Cultures infected with recombinant adenoviruses expressing RAFTK/pyk2 (WT), RAFTK/pyk2 (Tyr^{402}), or β-gal as a control were evaluated for apoptotic signaling. DNA fragmentation assessed by agarose gel electrophoresis was clearly increased in cultures expressing RAFTK/pyk2 (WT) but not substantially enhanced by the Tyr^{402} mutant (Fig. 2*A*). RAFTK/pyk2 (WT) expression induces a 2-fold increase in cleaved PARP relative to β-gal, whereas total PARP was reduced by 43% (Fig. 2*B*). Content of cleaved caspase-3 was also increased by RAFTK/pyk2 adenovirus over 3.5-fold compared with control β-gal infection (Fig. 2*C*). Participation of caspase-3 in RAFTK/pyk2-induced apoptotic signaling was confirmed by preemptive pharmacologic inhibition with Z-DEVD-cmk or Z-DMQD-CHO (caspase-3 inhibitor IV), which prevented DNA cleavage (Fig. 2*D*). Collectively, these results support the conclusion that sustained increases in RAFTK/pyk2 activity are pro-apoptotic involving a caspase-3 dependent signaling pathway.

RAFTK/pyk2 Activity Increases Phosphorylation of Src—Src kinase is activated by RAFTK/pyk2 via interaction with the Tyr^{402} residue and phosphorylation of Src at Tyr^{416} is indicative of activation. Immunoblots demonstrate marked increases in levels of RAFTK/pyk2 protein following infection with adenoviruses expressing RAFTK/pyk2 (WT) or the $Tyr(P)^{402}$ mutant (Fig. 3 *upper panel*). Immunoblotting for phosphotyrosine shows elevation of the phosphoprotein signal at 116 kDa in cultures expressing RAFTK/pyk2 consistent with the mobility of this kinase (Fig. 3, *middle panel* at *arrow*). It is tempting to speculate that the higher molecular weight phosphotyrosine immunoreactive band present in all samples is FAK based upon mobility at ~125 kDa and the loss of signal in the presence of RAFTK/pyk2 overexpression consistent with results shown later (see Fig. 5). The expression of RAFTK/pyk2 but not the $Tyr(P)^{402}$ mutant led to increased immunoreactivity for Tyr^{416} on Src consistent with activation of the kinase. Collectively these results indicate that RAFTK/pyk2 but not the $Tyr(P)^{402}$ mutant is capable of enhancing Src phosphorylation following overexpression.

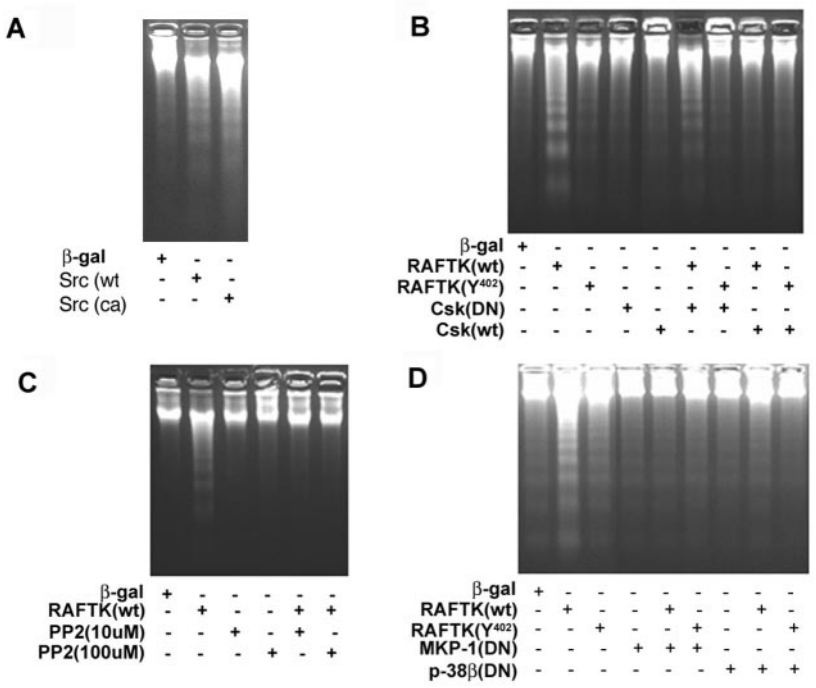

FIG. 4. **Src and p38 kinases mediate RAFTK/pyk2 apoptosis.** *A*, accumulation of Src (WT) or constitutively activated Src (ca) promote DNA laddering in cardiomyocyte cultures. *B*, Csk inhibits DNA laddering induced by RAFTK/pyk2. *C*, inhibition of Src by 2-h pretreatment with PP2 (0–100 μM) prevents DNA laddering mediated by subsequent challenge with RAFTK/pyk2. *D*, inhibition of MAPK activity by expression of dominant negative MKP-1 or p38β constructs prevents DNA laddering mediated by subsequent challenge with RAFTK/pyk2.

pyk2/RAFTK-induced Apoptosis Involves Src-dependent Signaling Cascades—Activation of Src by RAFTK/pyk2 raises the possibility that Src kinase participates in RAFTK/pyk2-mediated apoptosis. The role of Src as a mediator of apoptotic signaling by RAFTK/pyk2 was confirmed by adenoviral-mediated expression of Src kinase (WT and constitutively activated, Fig. 4*A*), expression of C-terminal Src kinase (Csk), a negative regulatory kinase of Src family tyrosine kinases (Fig. 4*B*), and pharmacologic treatment with PP2, an Src-specific tyrosine kinase inhibitor (Fig 4*C*). Overexpression of Src induced DNA fragmentation in cardiomyocyte cultures consistent with induction of apoptosis (Fig. 4*A*). Furthermore, DNA laddering was diminished by Csk (WT) accumulation in cultured cells prior to subsequent challenge infection with RAFTK/pyk2 (WT) adenovirus the following morning (Fig. 4*B*). In comparison, expression of a Csk dominant negative mutant failed to inhibit DNA laddering. Consistent with previous results (Fig. 2), infection with the RAFTK/pyk2 (Tyr^{402}) mutant had negligible effects upon DNA laddering in all experiments (Fig. 4). Similar to findings for Src inhibition, DNA laddering was also diminished by inhibition of p38 signaling by dominant negative mutants of either p-38β isoform or MKP-1 prior to subsequent challenge infection with RAFTK/pyk2 (WT) adenovirus the following morning (Fig. 4*D*). Taken together, results shown in Fig. 4 delineate a cascade of apoptotic signaling triggered by RAFTK/pyk2 activity leading to cell death that is antagonized by inhibition of Src kinase.

RAFTK/pyk2 Activity and Loss of Focal Adhesion-associated Proteins Is Restored by Paxillin Overexpression—RAFTK/pyk2-mediated induction of remodeling (Fig. 1*B*) suggests that focal adhesions may be affected, so immunoblotting was performed to determine expression levels of focal adhesion-associated proteins. Protein content of FAK, paxillin, and vinculin were all significantly reduced (29, 75, and 33%, respectively) following RAFTK/pyk2 (WT) expression relative to lysates from control cells expressing β-gal (Fig. 5*A*). In comparison, RAFTK/pyk2 (Tyr^{402}) with impaired Src binding lowered paxillin level by only 38% suggesting that RAFTK/pyk2-mediated loss of paxillin is augmented by Src activity. Effects upon FAK and vinculin were comparable between RAFTK (WT) and $Tyr(P)^{402}$, indicating that Src does not play a major role in these alterations. Together, these results indicate that activation of RAFTK/pyk2 leads to loss of focal adhesion proteins and paxillin content that may be important to focal adhesion stability. Cultured cardiomyocytes infected with paxillin (WT) or mutant (Tyr^{31}-Tyr^{118}) show normal myofibril organization with accumulation of paxillin throughout the cytoplasm (Fig. 5*B*). Cells expressing paxillin overnight resist characteristic loss of myofibril structure normally induced by RAFTK/pyk2 (Figs. 1*B* and 5*B*) upon subsequent challenge infection with RAFTK/pyk2 adenoviruses the following morning (data not shown). Preemptive 4-fold increases in expression levels of paxillin WT or mutant (Tyr^{31}-Tyr^{118}) also prevented decreases in FAK or vinculin protein content upon subsequent challenge infection with RAFTK/pyk2 adenoviruses the following morning (Fig. 5*C*), unlike cells expressing RAFTK/pyk2 alone (Fig. 5*A*).

Paxillin Phosphorylation Is Increased by RAFTK/pyk2 (WT) but Not Tyr^{402} Mutant—Enhanced kinase activity following expression of the RAFTK/pyk2 (WT) construct is shown by enhanced phosphorylation of paxillin, a known target substrate. Phosphorylation levels of paxillin residues Tyr^{31} and Tyr^{118} were increased 12.4- and 16.5-fold, respectively, relative to control levels (Fig. 6*A*), confirming these residues as targets for RAFTK/pyk2 activity in our system. In contrast, infection with the mutant RAFTK/pyk2 (Tyr^{402}) produced negligible changes in paxillin phosphorylation levels (Fig. 6*A*). Inhibition

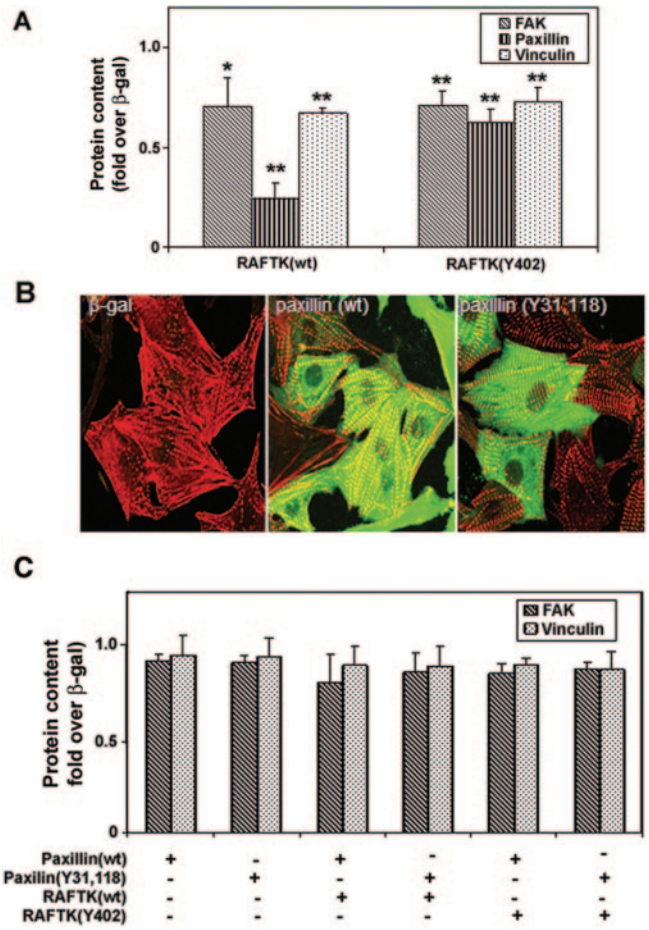

FIG. 5. **RAFTK/pyk2-mediated loss of focal adhesion proteins is restored by paxillin overexpression.** *A*, quantitation of immunoblot analysis for total protein content of culture lysates following infection with either RAFTK/pyk2 (WT) or the RAFTK/pyk2 (Tyr^{402}) mutant construct. Both RAFTK/pyk2 constructs show comparable decreases in FAK and vinculin, whereas the decrease in paxillin is markedly enhanced by RAFTK/pyk2 (WT) relative to the Tyr^{402} mutant construct. Signal intensities were corrected for effects of adenoviral infection by standardization to β-gal controls followed by correction for minor loading variations by normalization to GAPDH signal. **, $p < 0.01$ and *, $p < 0.05$ *versus* β-gal infection. *B*, confocal micrographs of neonatal cardiomyocytes expressing β-gal, paxillin (WT), or paxillin (Tyr^{31}-Tyr^{118}). Immunoreactivity with antibody to paxillin (*green*) is low in cells expressing β-gal but is readily visible in cells expressing either paxillin (WT) or paxillin (Tyr^{31}-Tyr^{118}). Phalloidin labeling (*red*) reveals normal sarcomeric organization in all infected cells undisturbed by paxillin accumulation. *C*, quantitation of immunoblots for FAK or vinculin content in culture lysates following infection with paxillin constructs either alone on in combination with RAFTK/pyk2 constructs. Paxillin content is markedly increased in all cultures, and subsequent challenge with RAFTK constructs does not significantly decrease FAK or vinculin protein levels. Signal intensities were corrected for effects of adenoviral infection by standardization to β-gal controls followed by correction for minor loading variations by normalization to GAPDH signal.

of paxillin phosphorylation was tested using a dominant negative paxillin construct with mutations at Tyr^{31} and Tyr^{118} residues (Fig. 6*B*). Infected cardiomyocytes showed accumulation of paxillin predominantly in the cytoplasm with 4-fold higher expression level relative to β-gal expression. Infection with RAFTK/pyk2 (WT) alone or in combination with paxillin (WT) showed 10–15-fold increases in phosphorylation of Tyr^{31} and Tyr^{118} residues. RAFTK/pyk2-mediated paxillin phosphorylation level at Tyr^{31} and Tyr^{118} residues was returned to basal levels by concurrent expression of the dominant negative paxillin protein. Impaired phosphorylation mediated by the RAFTK/pyk2 (Tyr^{402}) mutant construct was still apparent in

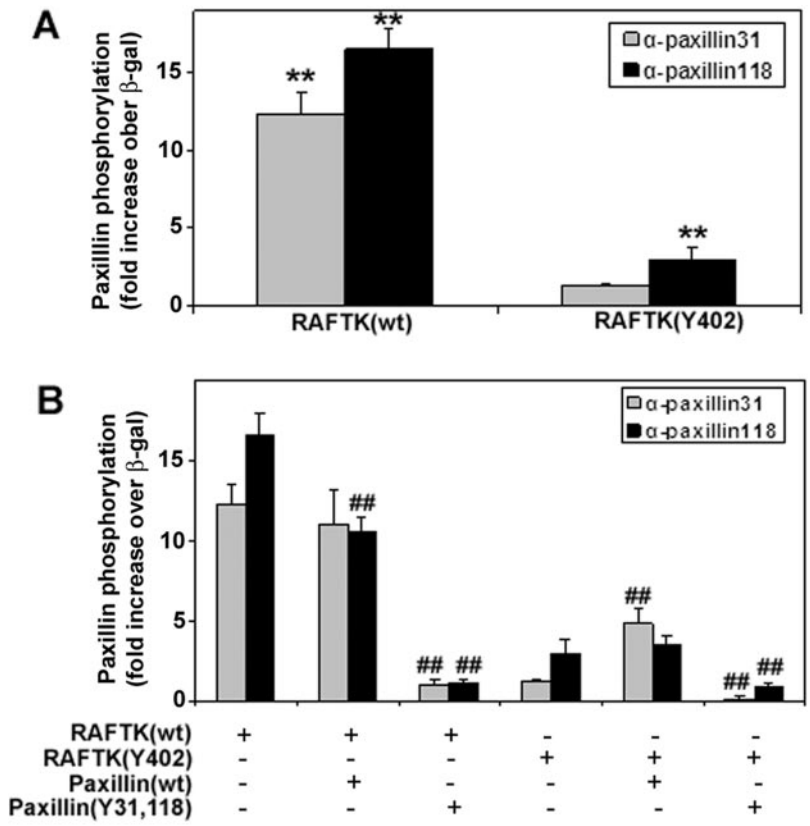

FIG. 6. **Overexpression of RAFTK/pyk2 affects paxillin phosphorylation.** Paxillin phosphorylation is mediated by RAFTK/pyk2 (WT) but not the RAFTK/pyk2 (Tyr^{402}) mutant construct. *A*, RAFTK/pyk2 (WT) phosphorylates endogenous paxillin or adenovirally encoded paxillin (WT), whereas a phosphorylation-deficient mutant lacks immunoreactivity at Tyr^{31} and Tyr^{118} residues. In comparison, the RAFTK/pyk2 (Tyr^{402}) mutant construct fails to increase paxillin phosphorylation. Signal intensity corrections made relative to cultures infected with adenovirus expressing β-gal as a negative control and minor loading were corrected by standardization *versus* GAPDH. Additionally, in *B* after normalization to GAPDH and β-gal, co-infection experiments were normalized to signals from cultures infected with either paxillin (WT) or paxillin (Tyr^{31}-Tyr^{118}) as appropriate. **, $p < 0.01$ *versus* β-gal infection; ##, $p < 0.01$ co-infections *versus* RAFTK/pyk2 (WT) or RAFTK/pyk2 (Tyr^{402}).

these experiments (Fig. 6*B*) although a noticeable rise in Tyr^{31} and Tyr^{118} phosphorylation was evident in coinfections together with paxillin (WT), suggesting that the abundance of paxillin substrate may allow for increased substrate phosphorylation by the mutant RAFTK/pyk2 (Tyr^{402}). Collectively, these experiments establish the characteristics of interaction between RAFTK/pyk2 and paxillin in both WT and mutated forms in cardiomyocyte cultures and demonstrate the capacity of RAFTK/pyk2 to alter paxillin phosphorylation levels.

Paxillin Overexpression Antagonizes RAFTK/pyk2-induced Apoptotic Signaling—Inhibition of RAFTK/pyk2-mediated effects by paxillin (Fig. 5) prompted experiments to determine anti-apoptotic effects mediated by paxillin in response to RAFTK/pyk2 infection. Consistent with these protective effects, cleavage of apoptosis-associated molecules including PARP and caspase-3 as well as DNA laddering were both diminished by paxillin accumulation in cultured cells prior to subsequent challenge infection with RAFTK/pyk2 (WT) adenovirus (Fig. 7, *A* and *B*). In all cases, the effect showed a tendency toward stronger protection by the double mutant Tyr^{31}-Tyr^{118} paxillin relative to the WT construct, but this difference was not statistically significant. Finally, paxillin-mediated protection is not a generalized anti-apoptotic effect because overnight accumulation of paxillin fails to inhibit DNA laddering induced by staurosporine (Fig. 7*C*). Together, these results indicate that paxillin accumulation inhibits pyk2/RAFTK-induced apoptotic signaling.

RAFTK/pyk2-mediated Increases in Phosphorylation of JNK and p38 Are Antagonized by Paxillin—Prior accumulation of paxillin inhibits the phosphorylation of JNK and p38 (Fig. 8), indicating that the activation of these kinases can be suppressed by paxillin. Prevention of JNK and p38 kinase phosphorylation by pre-emptive paxillin accumulation is consistent with protective anti-apoptotic effects mediated by paxillin accumulation (Fig. 7). Inhibitory effects comparable with paxillin (WT) were observed using the mutant paxillin (Tyr^{31}-Tyr^{118}, data not shown). Collectively, these results are consistent with RAFTK/pyk2 and paxillin being reciprocally involved in cell survival signaling that is mediated by connection through activation of downstream kinases.

DISCUSSION

Three novel findings to emerge from this study are that: 1) high level chronic activation of RAFTK/pyk2 promotes apoptotic signaling in cardiomyocytes, 2) RAFTK/pyk2-mediated apoptosis is mediated via Src kinase activation in cardiomyocytes, and 3) paxillin antagonizes RAFTK/pyk2-mediated effects. These observations establish a new dynamic relationship between apoptotic signaling and the cytoskeleton. In addition, downstream signal transduction stimulated by RAFTK/pyk2 expression reveals a previously unknown apoptotic cascade involving Src. The present results and conclusions provide evidence of the juxtaposition between calcium-activated kinase signal transduction against cytoskeletal-based signaling in cardiomyocyte survival.

Results of our study are consistent with apoptotic signaling mediated through a hub of Src kinase, placing this molecule at the center of a signal transduction network schematized in Fig. 9. RAFTK/pyk2 is the inciting stimulus for Src and also increases paxillin phosphorylation, which may be involved in reducing cellular paxillin content (Fig. 9 at the *question mark*). Loss of paxillin also diminishes interaction between paxillin and Src, exacerbating Src-dependent activation of JNK and p38β leading to cleavage of caspase-3 and promotion of apoptotic signaling. The complexity of the interdependent signaling in our system prevents separating anti-apoptotic effects of paxillin in the cytoskeletal (*e.g.* structural) *versus* cytoplasmic (*e.g.* signaling) domain. In fact, such subcellular fraction distinctions may be moot in view of the role as paxillin as target substrate and/or binding site for localization that helps to bring many of these molecules in proximity for coordinate regulation. The importance of paxillin as an adaptor docking scaffold to

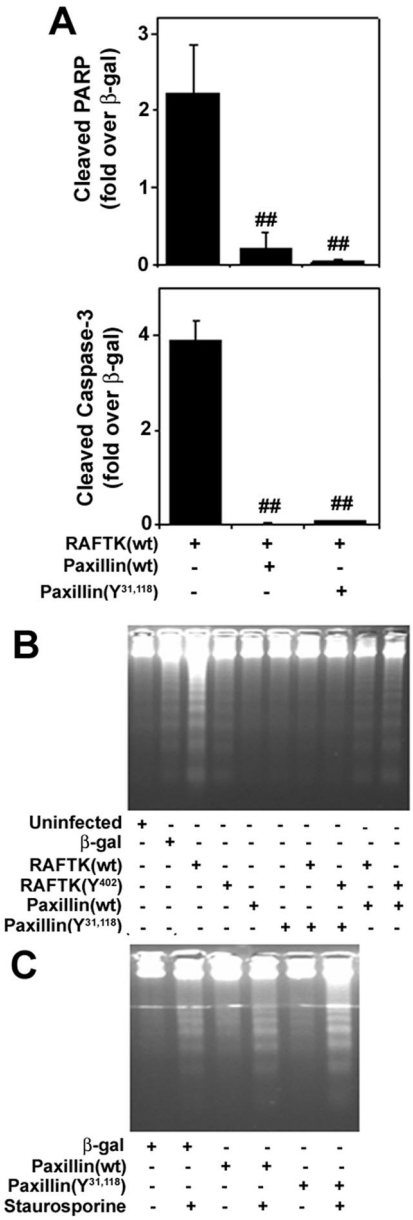

FIG. 7. **Paxillin accumulation antagonizes RAFTK/pyk2-induced apoptotic signaling and is ineffective in response to challenge with staurosporine.** *A*, paxillin overexpression prevents PARP and caspase-3 cleavage. -Fold changes were determined relative to signals from cultures infected with either paxillin (WT) or paxillin (Tyr^{31}-Tyr^{118}) alone as appropriate after normalization to GAPDH and β-gal infected controls. *B*, paxillin overexpression inhibits DNA laddering. *C*, anti-apoptotic effect of paxillin is ineffective in response to challenge with staurosporine. Cardiomyocytes were infected with adenoviruses expressing β-gal, paxillin (WT), or paxillin (Tyr^{31}-Tyr^{118}) and challenged with 1 μM staurosporine 24 h later. ##, $p < 0.01$ co-infections *versus* RAFTK/pyk2 (WT).

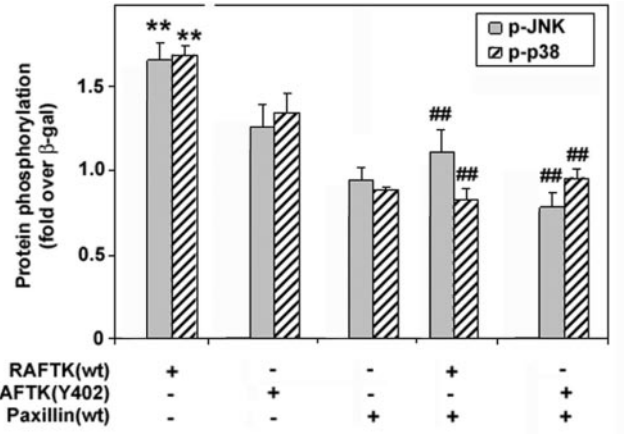

FIG. 8. **Increased phosphorylation of JNK and p38 MAPK by RAFTK/pyk2 constructs is inhibited by paxillin.** Quantitation of immunoblot for phosphorylation of JNK and p38 is shown. RAFTK/pyk2 (WT) increases phosphorylation of both kinases more than the dominant negative RAFTK/pyk2 (Tyr^{402}) mutant. Preemptive accumulation of paxillin (WT) protein inhibits phosphorylation of the kinases upon subsequent challenge with RAFTK/pyk2 constructs (shown in combination infections of last two groups). Each band was normalized to control infection with β-gal, and minor loading variations were corrected by standardization to GAPDH. First two infections with RAFTK/pyk2 constructs are **, $p < 0.01$ *versus* β-gal infection; ##, $p < 0.01$ co-infections *versus* RAFTK/pyk2 (WT) or RAFTK/pyk2 (Tyr^{402}).

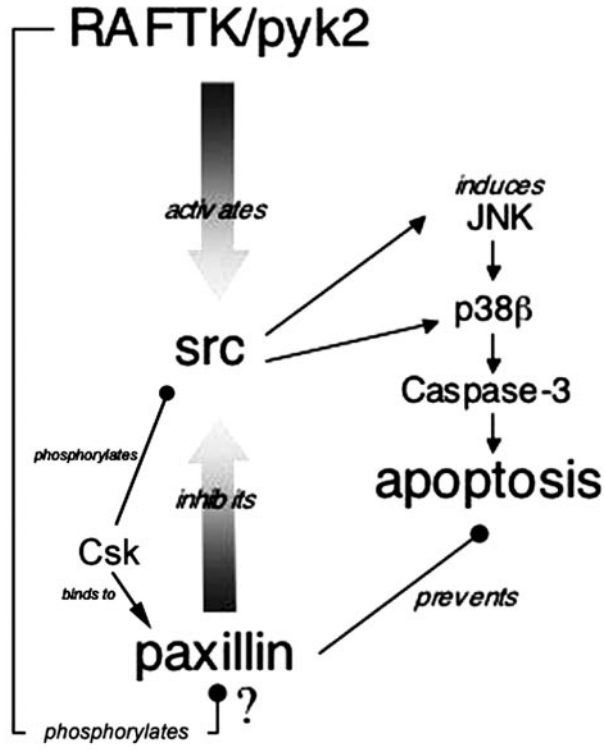

FIG. 9. **Paxillin antagonism of RAFTK/pyk2-mediated apoptotic signaling.** Hypothetical schematic representation depicting the central role of Src kinase as a mediator of apoptotic signaling in response to elevation of RAFTK/pyk2 activity. Chronic elevation of intracellular calcium levels leads to RAFTK/pyk2-mediated phosphorylation of paxillin, which is presumed to antagonize paxillin-mediated protection although this remains to be proven (indicated by the *question mark*). Although not directly inhibitory, paxillin may facilitate Src inhibition by acting as a docking site to bring Csk into proximity with Src. Positive feed-forward effects are indicated by *arrowheads*, whereas inhibitory effects are indicated by the *closed circles*.

reduce Src activity was postulated a decade ago (32); this reduction is possibly caused by bringing Src into close proximity with Csk to diminish Src activity (29, 30, 33). As such, loss of paxillin appears to remove constraints from RAFTK/pyk2-mediated signaling, promote Src activity, and initiate apoptosis. The balance between RAFTK/pyk2 and paxillin is an important starting point for future consideration of relationships between protein tyrosine kinase signaling, maintenance of cytoskeletal organization, and cellular survival.

RAFTK/pyk2 overexpression leads to enhanced autophosphorylation as well as increased paxillin phosphorylation that depends upon the critical Src binding site Tyr^{402} residue of RAFTK/pyk2 (Figs. 1 and 6, Ref. 34). Paxillin is a substrate for RAFTK/pyk2 (35), and activated RAFTK/pyk2 binds paxillin (36–39) concurrent with translocation of RAFTK/pyk2 to focal

adhesions (40). RAFTK/pyk2 activity and/or paxillin phosphorylation depends upon Src (35, 41) because Src kinase inhibitors prevent increased phosphorylation of RAFTK/pyk2 as well as paxillin (42). Our results are consistent with RAFTK/pyk2-mediated loss of paxillin promoting apoptotic signaling by promotion of Src kinase activity.

Src kinase mediates activation of mitogen-activated protein kinase family members. including stress-activated protein kinase/JNK and p38 kinases. JNK promotes apoptosis by phosphorylation and inhibition of pro-survival molecules (43–45) as well as direct activation of mitochondrial apoptotic machinery in adult cardiac myocytes (46). Tumor necrosis factor α-mediated activation of JNK leading to apoptosis in human neutrophils is decreased by inhibition of RAFTK/pyk2 (47). RAFTK/pyk2 also mediates apoptotic signaling via p38 activity in nonmyocyte cells (48, 49). In fact, apoptosis mediated by p38 is blocked by overexpression of dominant negative RAFTK/pyk2 (49). Prevention of DNA laddering by preemptive treatment with dominant negative mutants of either p38β or MKP-1 (Fig. 7*D*) confirms the participation of p38 in our system.

Paxillin-mediated survival signaling is enhanced by mutations in aspartic acids at caspase cleavage sites (50). Based upon our prior report of paxillin cleavage in failing hearts (7) we proposed that caspase-mediated cleavage of paxillin may contribute to the pathogenesis of heart failure. Caspase-mediated cleavage of paxillin has been linked to apoptosis (51), caspase activation leads to focal adhesion dissolution (52, 53), and caspase activity is associated with myocardial apoptosis and myofibrillar remodeling (54). Degradation of focal adhesion proteins is prevented by pretreatment with caspase inhibitors (55–57). Caspase activation leads to proteolytic cleavage of anchorage-mediated survival signaling elements such as Akt (58, 59), FAK (60, 61), β-catenin (62, 63), and p130CAS (64) that coordinate cell attachment with cell architecture, movement and gene expression. Induction of apoptosis by RAFTK/pyk2 is a novel finding for cardiomyocytes that is consistent with previous observations in other cell types (16, 65).

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Mechanisms of Signal Transduction:

Cardiomyocyte Apoptosis Triggered by RAFTK/pyk2 via Src Kinase Is Antagonized by Paxillin

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