Role of Protein-tyrosine Phosphatase SHP₂ in Focal Adhesion Kinase Down-regulation during Neutrophil Cathepsin G-induced Cardiomyocytes Anoikis*

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Inflammatory cells and their proteases contribute to tissue repair at site of inflammation. Although beneficial at early stages, excessive inflammatory reaction leads to cell death and tissue damage. Cathepsin G (Cat.G), a neutrophil-derived serine protease, has been shown to induce neonatal rat cardiomyocyte detachment and apoptosis by anoikis through caspase-3 dependent pathway. However the early mechanisms that trigger Cat.G-induced caspase-3 activation are not known. This study identifies focal adhesion kinase (FAK) tyrosine dephosphorylation as an early mechanism that regulates Cat.G-induced anoikis in cardiomyocytes. Both FAK tyrosine phosphorylation at Tyr-397 and kinase activity decrease rapidly upon Cat.G treatment and was associated with a decrease of FAK association with adapter and cytoskeletal proteins, p130Cas and paxillin, respectively. FAK-decreased tyrosine phosphorylation is required for Cat.G-induced myocyte anoikis as concurrent expression of phosphorylation-deficient FAK mutated at Tyr-397 or pretreatment with a protein-tyrosine phosphatase (PTP) inhibitor, pervanadate, blocks Cat.G-induced FAK tyrosine dephosphorylation, caspase-3 activation and DNA fragmentation. Analysis of PTPs activation shows that Cat.G treatment induces an increase of SHP₂ and PTEN phosphorylation; however, only SHP₂ forms a complex with FAK in response to Cat.G. Expression of dominant negative SHP₂ mutant markedly attenuates FAK tyrosine dephosphorylation induced by Cat.G and protects myocytes to undergo apoptosis. In contrast, increased SHP₂ expression exacerbates Cat.G-induced FAK tyrosine dephosphorylation and myocyte apoptosis. Taken together, these results show that Cat.G induces SHP₂ activation that leads to FAK tyrosine dephosphorylation and promotes cardiomyocyte anoikis.

One of the earliest events during the progression of cardiac failure is thought to involve an inflammatory response where inflammation and their proteases orchestrate myocardial repair. Although beneficial at early stages after myocardial injury, inflammatory cells release free radicals and proteolytic enzymes within the myocardium that may contribute to myocyte death and subsequent myocardial dysfunction (1, 2). Pathophysiologically relevant concentrations of inflammatory proteases have been shown to mimic many aspects of the phenotype in experimental animals, including cardiac dilatation and dysfunction, activation of fetal gene expression, cardiomoyocyte hypertrophy, and apoptosis. However, the mechanisms by which inflammatory proteases induce myocyte dysfunction are not well understood. Cathepsin G (Cat.G), a major serine protease released by activated neutrophils, has been proposed to play an important role in tissue remodeling at sites of wound-ing or tissue injury (3). Cat.G has been shown to hydrolyze a host of proteins including chemoattractants (4), extracellular matrix (ECM) (5, 6), and hormonal factors (7). However, recent studies have focused on the actions of Cat.G to cleave G protein-coupled protease-activated receptors as a mechanism whereby Cat.G modulates coagulation and tissue remodeling at sites of injury and inflammation (3, 8). In cultured neonatal rat cardiomyocytes (NRCMs), we have shown that Cat.G activates a signaling cascade (MAPK, AKT, and caspase-3) that culminates in myocyte detachment and apoptosis. The effect of Cat.G on myocytes was independent of protease-activated receptors activation, and speculation was raised about the role of Cat.G to hydrolyze ECM components that are critical mediators of normal cell-cell or cell-matrix interactions and are necessary for cell survival (9).

Cell adhesion to ECM through integrin receptors is critical for normal cell function (10). Perturbation of proper cell-ECM interactions is observed in various acute and chronic pathological conditions including ischemia/reperfusion injury (11). Loss of such interactions of normal cells results in the onset of apoptosis referred to as anoikis (12). Altered signaling that results from a loss of cell-ECM interactions is critical in this process, and focal adhesion kinase (FAK) seems to play an important role.
role (10, 11). Overexpression of constitutively active FAK prevents anoikis of many cells (13, 14), whereas inhibition or attenuation of FAK expression either by antisense oligonucleotides or expression of dominant-interfering mutant FRNK (FAK-related non-kinase) led to adhesion loss and subsequent apoptotic cell death of a variety of cell types including myocytes (15). However, whether endogenous FAK in adherent cells (the physiological situation) is critical for the control of myocyte survival and whether this is directly related to changes in the focal adhesion (FA) organization is still unclear.

Upon integrin binding to ECM, FAK is autophosphorylated on tyrosine residue 397, which is a docking site for the Src homology 2 (SH2) domain of Src family kinases (16). As a consequence of Src binding, other tyrosine residues of FAK, including Tyr-576, Tyr-861, and Tyr-925, are phosphorylated resulting in increased kinase activity of FAK and additional docking of other adapter and signaling molecules, including p130Cas, paxillin, phosphatidylinositol 3-kinase, and Grb2 (10). Thus, the coordinated activation of these complexes is likely to be critical in diverse cellular processes such as FA formation/turnover, cell spreading and migration, cell proliferation, and control of apoptosis.

Focal adhesion formation is a dynamic process in which assembly and disassembly are regulated by the balance between the activity of kinase enzymes and the phosphatases that catalyze the dephosphorylation reaction. Several intracellular protein-tyrosine phosphatases (PTPs) have been implicated as positive and negative regulators of integrin-mediated signaling (17, 18). Key signaling components such as FAK, p130Cas, and Src-protein-tyrosine kinases appear to be regulated by more than one PTP, probably reflecting the numerous signal inputs that can be integrated by this pathway. SHP2, PTP1B, PTP Pest, and PTEN all have been shown to modulate FA turnover by a direct action on FAK and p130Cas (18–21). Moreover, spreading and migration defects are observed in fibroblasts prepared from gene-targeted mice that have null or functionally null SHP2, PTEN, Cas, and Src (10). Evidence for an interplay between FAK and PTPs has also been reported. Thus, the above findings show that PTPs intersect with protein-tyrosine kinases at multiple points downstream of the integrins, and the ablated or increased PTP activity has profound effects on the integrin-mediated processes.

In the present study, we described that treatment of myocytes with Cat.G at concentrations that are likely to occur in area of inflammation in vivo (22–24) induces early loss of FAK tyrosine phosphorylation and activity followed by important perturbations in FA organization as well as FAK-mediated myocyte survival signaling. Our data show for the first time a perturbation in FA organization as well as FAK-mediated processes. We also show that Cat.G-induced FAK down-regulation is mediated by the pathway that we termed the FAK-SHP2-PTEN axis.

**Experimental Procedures**

Materials—Cat.G and okadaic acid were obtained from Calbiochem. Monoclonal antibodies to FAK, p130Cas, and paxillin were obtained from BD Biosciences, and polyclonal antibodies for FAK were from Santa Cruz Biotechnology. Phospho-FAK Tyr-397 and 4G10 monoclonal antibodies for phosphotyrosine were from Upstate Biotechnology. Polyclonal antibodies for phospho-SHP2, phospho-PTEN, and caspase-3 were from Cell Signaling. Polyclonal antibody for 20 S proteasome “core” subunits was from Biomol International. All other chemicals were from standard suppliers.

Myocyte Preparation—Cardiac myocytes were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described with minor modification (25). After 30 min of preplating (to eliminate adherent fibroblasts), cells were plated at a density of 160,000/cm² in 10% fetal bovine serum DMEM supplemented with 1 mmol/liter L-glutamine and antibiotic/antimycotic solution. Under these high density conditions, the myocytes form cell-cell contacts and display spontaneous contractile activity within 24 h of plating.

Subcellular Fractions Preparation—Treated myocytes were homogenized in Triton X-100 extraction buffer (100 mM Tris-HCl, pH 7.4, 2% Triton X-100) at 4 °C, containing 1:100 dilution of Sigma protease inhibitor mixture (P 8340) and phosphatase inhibitor mixtures I and II (P 2850 and P 5726) containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), aprotonin, leupeptin, bestatin, pepstatin-A, E-64 (trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane), cantharidin, bromotetramisole, microcin, sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole. Triton X-100-soluble and -insoluble components were prepared as described previously (26). Briefly, the homogenized extract was centrifuged at 16,000 × g, with the supernatant representing the detergent-soluble fraction (soluble). The pellet was re-extracted with 1 ml of Triton X-100 extraction buffer to remove remaining detergent-soluble proteins. Re-extraction was followed by centrifugation, removal of the supernatant, and final resuspension of the pellet (insoluble low spin pellet) in SDS-sample buffer. The supernatant from the first spin was spun again at 100,000 × g for 2.5 h to obtain a membrane pellet (insoluble high spin pellet), and the pellet fraction was processed as described before (26). To all the detergent-soluble fractions, equal volumes of SDS-sample buffer were added. All samples with SDS-sample buffer were boiled for 5 min for gel electrophoresis.

Expression of Adenoviral Vectors—Production of recombinant adenovirus expressing wild-type FAK (WT-FAK) and dominant negative FAK (Tyr-397-FAK, a mutant that lacks autophosphorylation/SRC homology SH2 binding site) was described elsewhere (27). FAK protein expression was driven by coinfection with adenovirus expressing the Tet transactivator Ad-TA as described (27). Cells were infected at 10, 25, or 25 plaque-forming units (pfu)/cell for Ad-TA or Ad-TA with Ad-WT-FAK or Ad-Tyr-397-FAK, respectively. Recombinant adenovirus wild-type SHP2 or dominant negative SHP2 polypeptide (DSH2, a truncated construct lacking the catalytic site and containing only the tandem SH2 domains of SHP2) were prepared as described previously (28). Adenoviral vectors were purified using a kit from Virapur and titrated using BD Adeno-X rapid titer kit (BD Bioscience). NRCMs were infected with adenovirus in DMEM for 2 h, then 5% fetal bovine serum DMEM was added, and cells were incubated for an additional 48 h. Serum-free DMEM/F-12 medium was changed 1 h before the start of the experiments.

Inhibition of apoptosis mediated by Tyr-397-FAK overexpression was mediated by 2 h treatment with 100 μM concen-
trations of broad specificity caspase inhibitor Z-VAD-FMK, and infections was performed 48 h after treatment.

**Immunoprecipitation and Immunoblot Analysis**—Extraction of proteins from cultured cells was performed as described previously (29). Cell extracts were clarified by centrifugation at 12,000 rpm, and the supernatants (1 mg of protein/ml) were subjected to immunoprecipitation with corresponding antibodies. After overnight incubation at 4 °C, protein A- or G-agarose beads were added and left for an additional 3 h. Immunocomplexes were then subjected to SDS-PAGE followed by Western blot analysis according to methods published previously or to the manufacturer’s instructions (29). Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

**In Vitro Kinase Assays**—FAK was immunoprecipitated with polyclonal FAK antibodies as described above except that the lysis buffer contained no SDS. The kinase reactions were done in kinase assay buffer containing 10 μCi of [γ-32P]ATP, 10 mmol/liter Tris-HCl, pH 7.4, 5 mmol/liter MnCl₂, 1 mmol/liter dithiothreitol, and 20 mmol/liter ATP for 20 min at 30 °C. Reactions were stopped by adding an equal volume of 2× SDS-PAGE sample buffer and boiling for 5 min. Samples were then separated by SDS-7.5% PAGE and dried gels were exposed to x-ray film.

**Immunofluorescence**—NRCMs were grown on fibronectin-coated glass coverslips, fixed with 4% paraformaldehyde, permeabilized in phosphate-buffered saline containing 0.2% Triton, and blocked with 1% bovine serum albumin. Cells were incubated with anti-FAK or anti-SHP2 antibodies overnight at 4 °C. After washes, cells were incubated with texas red phalloidin or anti-20 S proteasome followed by incubation with Alexa conjugated anti-mouse (rabbit) Ig antibodies. After final washes and mounting, cells were examined using an epifluorescence microscope (Leica, Bannockburn, IL).

**Caspase-3 Assay**—Caspase-3 activity was measured with the CaspACE assay system (Promega, Madison, WI). In brief, myocyte lysates were prepared by Dounce homogenization in lysis buffer provided with the kit. The lysates were centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatants containing 100 μg of protein were used for caspase-3 assay. Caspase-3 activity was examined by measurement of the rate of cleavage of fluorogenic conjugated substrate (7-methoxy-coumarin-4-yl)acetyl-Val-Asp-Gln-Met-Asp-Gly-Trp-Lys-(2,4-dinitrophenyl)-NH₂. The specificity of the assay was confirmed by addition of the specific caspase-3 inhibitor Z-DEVD-FMK in the reaction mixture at a concentration of 50 μM during the incubation.

**Apoptotic Cell Death ELISA**—A cell death detection ELISA kit (Roche Applied Science) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligonucleosomes fragments associated with apoptotic cell death.

**DNA Fragmentation**—Treated myocytes were lysed with lysis buffer (0.8 mM EDTA, pH 8.0, 8 mM Tris-HCl (TE, pH 8.0) and 4% SDS), and DNA was extracted from the lysed cells followed by incubation with 40 μg/ml proteinase K and 40 μg/ml RNase for 1 h at 37 °C. The DNA was precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ice-cold ethanol at −20 °C overnight. Finally, pellets were resuspended in TE buffer, and DNA concentrations were quantified from the absorbance at 260 nm. 6–8 μg of DNA samples were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

**Data Analysis**—All experiments were performed at least three times from three different cultures, and results are expressed as mean ± S.E. Statistical analysis was performed using one-way analysis of variance 1. Data were considered statistically significant if p was <0.05.

**RESULTS**

**Cathepsin G Induces FAK Dephosphorylation and Inactivation prior to Cell Morphology Changes and Detachment**—NRCMs undergo retractile morphological changes and detachment from the ECM upon Cat.G treatment (9). To investigate the mechanism(s) underlying this effect, we examined the effect of Cat.G treatment on the tyrosine phosphorylation state of FAK and correlated these with morphological changes. Upon Cat.G treatment, FAK was rapidly dephosphorylated, as determined by blotting with anti-phosphotyrosine antibodies. This occurred within 10–15 min, and FAK remained hypophosphorylated for over 30 min (Fig. 1A). This decrease in FAK tyrosine phosphorylation does not result from FAK degradation as full-length blot probing for FAK with a polyclonal antibody did not detect any effect of Cat.G on FAK cleavage at 15 and 30 min (Fig. 1B). However, a small amount of more rapidly migrating FAK immunoreactivity (whose size corresponds to the truncated FAK fragment generated via caspase cleavage) accumulates at 1 h after Cat.G treatment. Since the kinase activity of FAK is regulated by its level of tyrosine phosphorylation (30), we performed an in vitro kinase assay to measure FAK kinase activity after immunoprecipitation with FAK-specific antibody. As shown in Fig. 1C, FAK immunoprecipitated from cells treated with Cat.G for 5 and 15 min had reduced autophosphorylation activity compared with FAK from untreated cells. FAK can be tyrosine-phosphorylated on a number of tyrosine residues, including Tyr-397, that is required for its kinase activity. To assess whether Cat.G affect FAK Tyr-397 phosphorylation site, we performed Western blot analysis using one-way analysis of variance 1. Data were considered statistically significant if p was <0.05.

**Cathepsin G-induced FAK Down-regulation**

Cathepsin G-induced FAK Down-regulation via tyrosine-phosphorylated paxillin, two important components of FA that associate with and are phosphorylated by FAK (32), is also observed after Cat.G treatment (Fig. 1, E and F). To investigate whether p130cas and paxillin are also regulated by Cat.G stimulation, analysis of p130cas and paxillin tyrosine phosphorylation was performed. p130cas and paxillin tyrosine phosphorylation was decreased in response to Cat.G, and the kinetics of this decrease correlated with the kinetics of FAK decreased tyrosine phosphorylation upon Cat.G stimulation (Fig. 1, E and F). These data indicate that Cat.G negatively regulates the kinase activity of FAK and associated downstream signaling molecules.
Although CatG-induced FAK tyrosine dephosphorylation occurred within 10–15 min after CatG treatment, CatG-induced morphological changes and cardiomyocyte detachment were observed only 30 min after treatment with CatG and became more dramatic at 2 h of CatG stimulation (Fig. 2A). Consistent with the cellular morphological changes, a dramatic reorganization of FAK labeling was observed after 30 min of CatG treatment (Fig. 2, B and C). In control NRCMs, FAK staining was densely concentrated in the perinuclear regions but less markedly at the cellular periphery, where it was diffusely distributed as has been described previously (33, 34). Rhodamine-conjugated phalloidin, which labels sarcomeric actin, revealed the typical sarcomeric pattern of repetitive striations, with the labeled structure representing the actin array of two adjacent sarcomeres (Fig. 2B, panel a). Although FAK was detected close to myofilaments, its distribution pattern was not defined and no consistent superimposition to myofilaments was observed. After 30 min of CatG treatment, FAK immunostaining, which was mostly localized in the central regions at a higher density, appeared to scatter to the peripheral cytoplasm and was seen as clusters overlapping the regions stained with phalloidin (Fig. 2B, panel b). This ectopic localization of FAK and actin in clusters is reminiscent of protein undergoing degradation. Therefore, we tested whether FAK aggregates associate with the proteasome complex. Antiserum raised against the 20 S core particle of the proteasome complex was used to stain cells and the same perinuclear and peripheral cytoplasm pattern of punctate staining was respectively seen in both controls and myocytes treated with CatG (Fig. 2C). This indicates a colocalization of FAK and the proteasome complex and shows that FAK degradation by the proteasome occurs at basal conditions, where it may play a role in FAK turnover, and in response to CatG treatment, suggesting that tyrosine-dephosphorylated FAKs are targeted for degradation by the proteasome. Consistent with this finding, CatG-induced FAK degradation was inhibited when cells were pretreated with the proteasome inhibitor MG132 (data not shown). These results suggest that FAK-decreased tyrosine phosphorylation and inactivation might be causally involved in CatG-induced rearrangement of FA components that are necessary for the morphological changes observed in cardiomyocytes.

**FAK Overexpression Rescue Myocyte Apoptosis Induced by Cathepsin G—**To gain more insight into the role of FAK in CatG signaling, we conducted gain- or loss-of-function experiments, in which we transduced NRCMs with adenoviruses carrying WT-FAK or truncated form of FAK (Tyr-397-FAK, a
mutant that lacks autophosphorylation/SRC homology SH2 binding site). Infected cardiomyocytes showed accumulation of WT-FAK in the cytoplasm and FA (Fig. 2B, panel c) and increases of 5-fold over control TA-infected cells (Fig. 3A). Treatment with Cat.G did not significantly affect FAK distribution (Fig. 2B, panel d) or FAK tyrosine phosphorylation (Fig. 3B) compared with WT-FAK-infected controls. In comparison, myocytes infected with Tyr-397-FAK showed accumulation of FAK in the cytoplasm, myofibrils, and stress fibers (Fig. 2B, panel e) and increases of 3.5-fold over TA-infected cells (Fig. 3A). Treatment with Cat.G did not show significant reorganization of FAK labeling (Fig. 2B, panel f) or FAK tyrosine phosphorylation (Fig. 3B) compared with Tyr-397-FAK-infected controls. Cat.G also led to marked cardiomyocyte remodeling and loss of myofibril organization in TA-infected myocytes and overexpression of WT-FAK significantly attenuated Cat.G-induced loss of myofibrils (Fig. 2D). In contrast, Tyr-397-FAK overexpression promoted loss of myofibril organization in ~30% of infected myocytes (mostly in peripheral regions), and treatment with Cat.G did not have any additional effect on myofibril organization (Fig. 2D).

To assess whether decreased FAK phosphorylation led to the regulation of myocyte apoptosis induced by Cat.G, myocytes infected with recombinant adenovirus expressing WT-FAK, Tyr-397-FAK, or TA were evaluated for apoptotic signaling. There was an increase in both caspase-3 activity and DNA fragmentation (assessed by ELISA and agarose gel electrophoresis) in cells treated with Cat.G for 8 h indicating an increase in apoptosis (Fig. 3, C–E). WT-FAK overexpression reduced basal myocyte apoptosis compared with control Ad-TA-infected cells and attenuated myocyte apoptosis induced by Cat.G. In contrast, Tyr-397-FAK overexpression increased basal myocyte apoptosis.

**FIGURE 2.** FAK overexpression prevents Cat.G-induced FAK redistribution and myofibrillar disorganization. A, phase contrast photomicrographs of NRCM cultures treated with 400 nmol/liter Cat.G for the indicated time. Bar = 100 μm. B, NRCMs infected with Ad-TA (10 pfu/cell), WT-FAK (25 pfu/cell), or Tyr-397-FAK (25 pfu/cell) were untreated (panels a, c, and e) or treated with Cat.G (400 nmol/liter) (panels b, d, and f). After 30 min cells were fixed and stained for FAK (green) or phalloidin (red). Bar = 20 μm. C, NRCMs were untreated (panels a, c, and e) or treated with Cat.G (400 nmol/liter) (panels b, d, and f). After 30 min cells were fixed and stained for FAK (green) or 20 S proteasome (red). Bar = 20 μm. Note the colocalization of FAK and 20 S proteasome after Cat.G treatment. D, sarcomeric organization measured as the percentage of total cells displaying well developed sarcomeric banding patterns.
Cathepsin G-induced FAK Down-regulation

FIGURE 3. FAK overexpression rescue Cat.G-induced myocyte apoptosis. Lysates from myocytes infected with either TA (10 pfu/cell), WT-FAK (25 pfu/cell), or Y397-FAK (25 pfu/cell) adenoviruses for 48 h and either untreated or treated with Cat.G were assessed for Western blot using anti-FAK antibodies (A) or treated with Cat.G for 15 min followed by immunoprecipitation (IP) with anti-FAK antibodies (B). FAK tyrosine phosphorylation was determined by Western blot using anti-phosphotyrosine (P-Tyr) or anti-FAK antibodies. C, caspase-3 activity was measured using fluorogenic substrate as described under “Experimental Procedures.” Results are expressed as relative fluorescence unit (RFU)/min/mg for triplicate determinations from a single experiment (mean ± S.E.). D, DNA fragmentation as measured by anti-histone antibody ELISA. Results are expressed as relative DNA fragmentation (A410–A500). *p < 0.05 versus control; #p < 0.05 versus Cat.G-treated myocytes. E, cellular DNA (6–8 μg) was separated using 2% agarose gels and revealed by ethidium bromide staining. F and G, inhibition of caspase-3 cleavage by 2-h pretreatment with 100 μM Z-DEVD-CMK prevents caspase-3 activation (F) and DNA laddering (G) mediated by subsequent challenge with Tyr-397-FAK.

Cathepsin G-induced FAK Down-regulation

Cathepsin G-decreased FAK Tyrosine Phosphorylation and Apoptosis of Cardiomyocytes Are Protein-tyrosine Phosphatase-dependent—To examine whether decreased tyrosine phosphorylation of FAK induced by Cat.G is PTP-dependent, pervanadate, a general PTP inhibitor, was applied before Cat.G treatment. As shown in Fig. 4A, pretreatment with pervanadate blocked Cat.G-induced tyrosine dephosphorylation of FAK. Moreover, pretreatment with pervanadate prevented Cat. G-induced morphological changes and cardiomyocyte detachment, although treatment with pervanadate alone induced marked morphological changes in control myocytes (Fig. 4B). Pretreatment with pervanadate also prevented the increase in both caspase-3 activity and DNA fragmentation induced by Cat.G (Fig. 4, C–E). It is noteworthy that some basal variations in DNA fragmentation are noticeable between Figs. 3 and 4. These variations may be related to differences in the controls used in each figure (infected versus non-infected with TA adenoviruses) or related to variations that occur using ELISA as a cell death detection method. Taken together, these data indicate that PTP-mediated down-regulation of FAK activity is responsible for Cat.G-induced myocyte apoptosis.

Cathepsin G Induces SHP2 and PTEN Phosphorylation in Rat Cardiomyocytes—The SH2 domain-containing protein-tyrosine phosphatases SHP2 and PTEN can associate and dephosphorylate FAK and paxillin (18, 21). To explore the potential involvement of these phosphatases in Cat.G-mediated tyrosine dephosphorylation of FAK, we analyzed the effect of Cat.G on SHP2 and PTEN activation by Western blot analysis using antibodies that recognize the phosphoactive SHP2 at Tyr-542 and PTEN at Ser-380/Thr-382/383. Cat.G induced phosphorylation of both SHP2 and PTEN but with different kinetics. The activation of SHP2 was rapid and sustained, whereas the activation of PTEN occurred late, only 2 h after Cat.G treatment (Fig. 5, A and B). These data together suggest that SHP2 but not PTEN is likely involved in Cat.G-induced FAK tyrosine dephosphorylation. To analyze whether FAK tyrosine dephosphorylation observed in myocytes is a direct consequence of SHP2 activation, we analyzed FAK association to SHP2 and the result of this association on FAK tyrosine phosphorylation status. Immunoprecipitation studies showed that FAK and SHP2 complex formation increased after apoptosis compared with control Ad-TA-infected myocytes; however, it blocks caspase-3 activation and DNA fragmentation induced by Cat.G (Fig. 3, C–E). Participation of caspases in Tyr-397-FAK-induced apoptotic signaling was confirmed by preemptive pharmacologic inhibition with Z-DEVD-FMK (a cell-permeable general caspase inhibitor), which prevented caspase-3 activity and DNA cleavage (Fig. 3, F and G). These data show that while WT-FAK overexpression rescues myocyte from undergoing apoptosis by reducing both basal and Cat.G effect on myocyte apoptosis, Tyr-397-FAK overexpression plays an important role in mediating Cat.G-induced myocyte anoikis.

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Cat.G treatment (Fig. 5C). The FAK-SHP$_2$ complex was detected at 15 min and increased 30 min after Cat.G treatment. To delineate FAK and SHP$_2$ localization, control and Cat.G-treated myocytes were double immunostained with monoclonal anti-FAK and polyclonal anti-SHP$_2$ antibodies. The FAK immunolabeling showed normal FA structures, and SHP$_2$ staining was homogeneously distributed throughout the cytoplasm and nucleus in control cells, and treatment with Cat.G induced FAK and SHP$_2$ colocalization and their accumulation at high density in the cytoplasm (Fig. 5D).

To confirm FAK and SHP$_2$ codistribution after treatment with Cat.G, myocytes were lysed with Triton X-100-containing buffer and centrifuged to obtain low-spin (16,000 × g) and high spin (100,000 × g) pellets (actin-rich cytoskeleton and membrane skeleton, respectively) as well as low spin supernatant (soluble fraction) (Fig. 5E). The majority of FAK and SHP$_2$ expression was present in the Triton X-100-soluble fractions, just as has been reported previously (26). They were present at low levels in the Triton X-100-insoluble low and high spin fractions of control cardiomyocytes. In Cat.G-treated myocytes, FAK and SHP$_2$ translocation to the soluble fraction was found to be significantly increased and correlated with decrease in FAK and SHP$_2$ association with cytoskeleton and membrane fractions, respectively. As expected, actin was present at high levels in cytoskeleton and membrane skeleton fractions relative to soluble fractions, and treatment with Cat.G for 15 and 30 min induced a trivial increase of actin to soluble fraction. These experiments indicate that FAK and SHP$_2$ redistribute to the Triton X-100-soluble fraction upon Cat.G treatment.

**SHP$_2$ Catalytic Activity Is Involved in Cathepsin G-induced FAK Tyrosine Dephosphorylation**—To determine whether SHP$_2$ catalytic activity is required for Cat.G-induced FAK tyrosine dephosphorylation, NRCMs were infected with recombinant adenoviral vectors expressing wild-type SHP$_2$ (WT-SHP$_2$) or a mutated construct expressing catalytically inactive SHP$_2$ (DSH2). Infected myocytes with DSH2 at 10,
25, and 50 pfu/cell showed an increase level of truncated mutant SHP2 by 9-, 13-, and 15-fold, respectively over LacZ control, while the expression of the endogenous SHP2 decreased by 0.2-, 0.3-, and 0.5-fold, respectively, over control LacZ (Fig. 6A). DSH2 overexpression attenuated FAK tyrosine dephosphorylation induced by Cat.G with no detectable effect on FAK expression levels (Fig. 6C). Myocytes infected with WT-SHP2 viruses at 25, 50, and 100 pfu/cell showed an increased level of transgenic SHP2 expression by 0.2-, 0.4-, and 0.5-fold, respectively, over LacZ-infected controls as determined by Western blotting (Fig. 6B). Overexpression of WT-SHP2 led to a decrease in basal tyrosine phosphorylation of FAK compared with control myocytes infected with LacZ adenoviruses, and treatment with Cat.G did not significantly affect FAK tyrosine dephosphorylation (Fig. 6C). Taken together, these data demonstrate that Cat.G-induced SHP2 activation is involved in FAK tyrosine dephosphorylation in cardiomyocytes.
**DISCUSSION**

The present study demonstrates a role of FAK tyrosine dephosphorylation in Cat.G-induced cardiomyocyte apoptosis. FAK is rapidly dephosphorylated on the tyrosine residue, and its interaction with other adapter and cytoskeletal proteins (p130Cas and paxillin) is decreased after stimulation with Cat.G. Moreover, FAK interacts with SHP2, tyrosine phosphatase that is involved in its tyrosine dephosphorylation. Consistent with potential involvement of SHP2 in Cat.G signaling pathway, inhibition of SHP2 by recombinant adenovirus vector expressing DSH2 was sufficient to attenuate FAK tyrosine dephosphorylation and protected myocytes to undergo apoptosis after Cat.G treatment.

Although increased FAK tyrosine phosphorylation occurs in response to diverse extracellular stimuli, FAK maintains a relatively high basal level of phosphorylation and activity (35). The role of this constitutive phosphorylation in normal cellular function is not fully understood but may be important for maintaining cell survival signaling and FA integrity in the resting state. A major finding of the present paper is that physiopathological concentrations of Cat.G similar to that seen in area of inflammation in vivo (22–24) induced a potent and rapid tyrosine dephosphorylation of FAK in cardiomyocytes that was maximal within minutes and occurred concomitantly with disengagement of FAK from FA. This correlates with loss of FAK interaction with p130Cas and paxillin, which are important docking sites for other signaling molecules and play an additional role in survival signaling (10, 36). This is consistent with FAK involvement in the tyrosine phosphorylation of p130Cas and paxillin (10, 37, 38) and indicates that Cat.G induces a rapid down-regulation of FA signaling as well as destruction of the FA complex in cardiomyocytes. Therefore these effects of Cat.G occur before the earliest detectable increase in caspase-3 activation (2–4 h) and before the onset of detectable FAK proteolysis (Fig. 1B and Ref. 9). Indeed, we found an increase in FAK colocalization with the proteasome complex subunit 20 S at later stages after Cat.G treatment (Fig. 2C). These data together strongly suggest that whereas decreased expression and protoeolysis of FAK are late consequences of the terminal execution phase of Cat.G-induced apoptosis, loss of FAK tyrosine phosphorylation is an early trigger for disruption of the FA complex and loss of survival signals relayed through FA. Consistent with these findings, overexpression of WT-FAK was sufficient to reduce basal myocyte apoptosis and significantly decreased myocyte apoptosis induced by Cat.G. These data extend previous studies showing that transient overexpression of the FA targeting (FAT) domain or FRNK, a COOH-terminal inhibitor of FAK, induced apoptosis by loss of FA in NRCMs (15, 39).

Because FAK tyrosine phosphorylation controls its catalytic

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**FIGURE 6. SHP2 catalytic activity is involved in Cat.G-induced FAK tyrosine dephosphorylation.** NRCMs were infected at different pfu/cell ratios with recombinant adenoviral vectors expressing catalytically inactive SHP2 (DSH2) (A) or WT-SHP2 (B) for 48 h. Levels of SHP2 over- or down-expression in NRCMs were determined by immunoblotting with anti-SHP2 antibodies. C, cells infected with either LacZ (25 pfu/cell), DSH2 (25 pfu/cell), or WT-SHP2 (50 pfu/cell) adenoviruses for 48 h were treated with Cat.G (400 nmol/liter) for 15 min. Cell lysates were immunoprecipitated (IP) with anti-FAK antibodies followed by Western blot using anti-phosphotyrosine (P-Tyr) or anti-FAK antibodies. The figures are representative of results obtained in three separate cultures.

**SHP2 Inhibition Markedly Attenuates Cardiomyocyte Apoptosis Induced by Cathepsin G**—To assess whether SHP2-dependent tyrosine dephosphorylation of FAK led to the regulation of myocyte anoikis induced by Cat.G, myocytes infected with recombinant adenovirus expressing WT-SHP2, DSH2, or LacZ were evaluated for apoptotic signaling. DSH2 overexpression had no detectable effect on cell morphology in untreated myocytes; however, it reduced Cat.G-induced morphological changes and cardiomyocyte detachment (Fig. 7A). In contrast, WT-SHP2 overexpression increased myocyte detachment in untreated myocytes and enhanced the number of detached myocytes induced after Cat.G treatment. Caspase-3 activity and DNA fragmentation were also significantly increased after Cat.G treatment in LacZ-infected myocytes, and overexpression of DSH2 mutant, and not WT-SHP-2, markedly inhibited both caspase-3 and DNA fragmentation induced by Cat.G treatment (Fig. 7, B and C). We also noticed an increase in basal hallmarks of apoptosis in myocyte infected with WT-SHP2 adenoviruses compared with control LacZ-infected myocytes. These data together show that catalytic activity of SHP2 is both necessary and sufficient to promote Cat.G-induced myocyte anoikis.
activity and association with other signaling molecules, dephosphorylation of specific tyrosine residues on FAK is potentially a very important mechanism for the regulation of its functional state and for the activation of its downstream molecular pathways. Herein, Cat.G decreased FAK tyrosine phosphorylation at the Tyr-397 residue and overexpression of the Tyr-397-FAK mutant was sufficient to induce loss of myofibrillar organization and increase basal myocyte apoptosis demonstrating the requirement of Tyr-397 phosphorylation site in maintaining myocyte survival. However, deletion of this site significantly inhibited Cat.G-induced myocyte apoptosis suggesting that Tyr-397 site phosphorylation and dephosphorylation constitutes a molecular switch that controls myocyte survival and apoptosis, respectively. The best characterized function of FAK at Tyr-397 is the creation of a high affinity binding site for the SH2 domain of Src family members, which then phosphorylate other FAK tyrosine residues, increasing FAK activity (16). Phosphorylated Tyr-397 can also bind phosphatidylinositol 3-kinase and phospholipase Cγ (10), suggesting that FAK might be involved in lipid signaling and focal contact localization of second messengers, thereby influencing actin polymerization (40) and cytoskeletal organization during ECM assembly.

FAK tyrosine dephosphorylation may occur through indirect mechanism involving the COOH-terminal Src kinase (41), which inhibits Src-mediated FAK phosphorylation (16) or through direct activation of tyrosine phosphatases (19–21). In this study, we found that FAK tyrosine dephosphorylation induced by Cat.G was prevented by the PTPs inhibitor pervanadate pretreatment, which also prevented Cat.G-induced myocyte apoptosis. To investigate which PTPs are likely to mediate Cat.G-induced FAK tyrosine dephosphorylation, we found that activation of SHP2 and PTEN, two PTPs known to be directly or indirectly responsible for FAK and p130Cas tyrosine dephosphorylation (18, 21), was enhanced after Cat.G treatment but with different kinetics. The fact that PTEN activation is late (occurs only 30 min-2 h after Cat.G treatment) does not correlate with FAK tyrosine dephosphorylation (compare Fig. 1A and Fig. 5B) and that PTEN does not physically interact with FAK (data not shown) strongly rules out any involvement of PTEN in mediating FAK tyrosine dephosphorylation that occurs after Cat.G treatment, although a role of PTEN at later stages of Cat.G-induced cardiomyocyte apoptosis cannot be discarded. In contrast, the involvement of SHP2 in FAK tyrosine dephosphorylation induced by Cat.G treatment is supported by several lines of evidence. First, SHP2 activation occurs early (5 min after Cat.G treatment), and it is associated with an increase in SHP2/FAK interaction. Second, both FAK and SHP-2 codistribute in the cytosolic fraction after Cat.G treatment as shown by both immunocytochemistry and subcellular fractionation experiments. Third, DSH2 mutant expression significantly inhibited Cat.G-induced FAK tyrosine

**FIGURE 7.** SHP2 inhibition markedly attenuates cardiomyocyte apoptosis induced by Cat.G. A, phase contrast photomicrographs of NRCM cultures untreated or treated with Cat.G for 2 h. Bar = 100 μm. B, caspase-3 activity was measured using fluorogenic substrate. Results are expressed as relative fluorescence unit (RFU)/min/mg for triplicate determinations from a single experiment (mean ± S.E.). C, DNA fragmentation as measured by anti-histone antibody ELISA. Results are expressed as relative A410/A500. *p < 0.05 versus control; #, p < 0.05 versus Cat.G-treated myocytes.
dephosphorylation and attenuated Cat.G-induced myocyte apoptosis. Fourth, expression of WT-SHP2 was sufficient to decrease FAK tyrosine phosphorylation and enhanced Cat.G-induced myocyte apoptosis. These findings extend previous studies showing the involvement of SHP2, in the turnover of FA, decreasing FAK tyrosine phosphorylation and enhanced Cat.G-induced apoptosis may depend on the potency and/or duration of SHP2 involvement on either cardiomyocyte hypertrophy or apoptosis induced by Cat.G treatment. This suggests that PTPs (other than SHP2) are involved in regulating FAK phosphorylation and activation that FAK tyrosine phosphorylation is under other control mechanisms. Indeed, indirect mechanisms involving the COOH-terminal Src kinase or phosphorylation of serine residues of FAK has been shown to affect negatively FAK tyrosine phosphorylation (41, 43, 44). On the other hand, SHP2 also becomes tyrosine-phosphorylated in response to various stimuli (21, 45–47) and may mediate cardiomyocyte hypertrophy (47). Although the significance of this discrepancy is unclear, one can propose that SHP2 activation and its involvement on either cardiomyocyte hypertrophy or apoptosis may depend on the potency and/or duration of SHP2 activation and may be context-dependent.

The ability of Cat.G to release myocytes from normal anchorage-dependent binding to ECM to an anchorage-independent status could be an important signal in triggering apoptosis during some physiopathological conditions (i.e. myocardial infarction). Although the role of anoikis in cardiac remodeling is largely unknown, studies by Ding et al. (48) were the first to emphasize that cardiomyocyte anoikis may be responsible for the slight increases in myocyte apoptosis observed during the transition from cardiac hypertrophy to heart failure when abnormal myocyte contacts with the ECM were observed. Alteration of cellular contacts with the ECM also has been shown to result in the shedding of a β1-integrin fragment from the cell surface during aortic stenosis-induced cardiac hypertrophy (49). These data together suggest that the pro-apoptotic effects of Cat.G on cardiomyocytes may play a critical role in area of inflammation, such as during myocardial infarction, where neutrophil infiltration has been shown to play an important role in cardiomyocyte death and extension of infarction. The studies showing the cardioprotective effect of serine proteases inhibitor LEX032 (a recombinant serine protease inhibitor of neutrophil Cat.G and elastase) after ischemia/reperfusion suggest that Cat.G may be a particularly important mediator of cardiac injury (50).

In conclusion, these data indicate an important role of FAK tyrosine phosphorylation in the control of neutrophil derived serine protease induced apoptosis; loss of FAK activity caused by Cat.G results in perturbations of FA organization with a subsequent inactivation of associated signaling molecules and loss of survival signaling. Our observation that SHP2 activation is involved in FAK tyrosine dephosphorylation and myocyte apoptosis suggests a potential role for PTPs in the regulation of cell-substratum adhesion after Cat.G treatment.

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