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p38γ MAPK contributes to left ventricular remodeling after pathologic stress and disinhibits calpain through phosphorylation of calpastatin

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ABSTRACT: Despite the high and preferential expression of p38γ MAPK in the myocardium, little is known about its function in the heart. The aim of the current study was to elucidate the physiologic and biochemical roles of p38γ in the heart. Expression and subcellular localization of p38 isoforms was determined in mouse hearts. Comparisons of the cardiac function and structure of wild-type and p38γ knockout (KO) mice at baseline and after abdominal aortic banding demonstrated that KO mice developed less ventricular hypertrophy and that contractile function is better preserved. To identify potential substrates of p38γ, we generated an analog-sensitive mutant to affinity tag endogenous myocardial proteins. Among other proteins, this technique identified calpastatin as a direct p38γ substrate. Moreover, phosphorylation of calpastatin by p38γ impaired its ability to inhibit the protease, calpain. We have identified p38γ as an important determinant of the progression of pathologic cardiac hypertrophy after aortic banding in mice. In addition, we have identified calpastatin, among other substrates, as a novel direct target of p38γ that may contribute to the protection observed in p38γKO mice.—Loonat, A. A., Martin, E. D., Sarafraz-Shekary, N., Tilgner, K., Hertz, N. T., Levin, R., Shokat, K. M., Burlingame, A. L., Arabacilar, P., Uddin, S., Thomas, M., Marber, M. S., Clark, J. E. p38γ MAPK contributes to left ventricular remodeling after pathologic stress and disinhibits calpain through phosphorylation of calpastatin. FASEB J. 33, 13131–13144 (2019). www.fasebj.org

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The current study provides experimental evidence for a putative role for p38γ MAPK in the progression of cardiac hypertrophy and heart failure and elucidates a mechanism of action. By using an in vivo mouse model of cardiac hypertrophy, this study demonstrates how ablation of p38γ MAPK expression ameliorates cardiac function after aortic constriction. By using a modified analog-sensitive kinase in vitro and subsequent proteomic analysis, we identify putative substrates of p38γ MAPK and demonstrate the functional consequence of substrate phosphorylation.

p38 MAPKs are stress-activated serine/threonine kinases that sense a variety of cardiac pathologies (1). The family consists of 4 isoforms—a, β, γ, and δ. Studies have historically focused on the p38α and p38β isoforms as they are ubiquitously expressed and inhibited by widely available tools, including pyridynylimadazole inhibitors, such as SB203580. The remaining 2 p38 MAPK isoforms are not inhibited by pyridynylimidazoles and exhibit differential expression patterns (2). For example, p38γ is highly expressed in skeletal and cardiac muscle, and p38δ is highly expressed in endocrine glands (3).

Whereas all 4 isoforms are expressed in the heart, p38α and p38γ have been shown to be the 2 most abundantly expressed isoforms, with comparable myocardial protein content (3). There are extensive studies that have documented the role of p38α in various cardiac disease states, especially in response to ischemia and infarction (1); however, little has been reported about the functional role of p38γ in the heart. Given the differential localization of p38α and p38γ in unstimulated cardiomyocytes and in...
hearts of mice subjected to pressure overload (3, 4), it is unlikely that p38y is functionally redundant.

The lack of tool compounds has also impeded the identification of p38y substrates. The PDZ domain-interacting motif—exclusive to p38y among the MAPK family—has aided in the search for specific substrates (5). The short amino acid sequence, KETAL, in the C-terminal of p38y can directly dock to PDZ domains of proteins and therefore adds a unique dimension to p38y signaling. Examples of proteins that have been found to interact with p38y via their PDZ domains and undergo phosphorylation are α-1-syntrophin (5), PSD-95 (6), SAP97 (7), PTPHI (8), and DEPTOR (9); however, there are likely to be cardiac substrates of p38y that are not PDZ dependent. One technique to identify such substrates is an in vitro chemical genetic approach first described by Shokat and colleagues (10). This technique was initially developed to identify substrates of v-Src tyrosine kinase. The approach requires the generation of an analog-sensitive (AS) kinase that can utilize bulky, orthogonal N6 expanded ATP analogs that cannot be used by wild-type (WT) kinases. The ATP analog is also modified at the γ-phosphate to substitute a thiol group in place of the hydroxyl group (11). As such, substrate proteins undergo thiophosphorylation, which allows substrates of the kinase of interest to be specifically tracked and identified from a complex mixture of proteins, such as cell or tissue extracts.

The aim of the current study was to examine the role that p38y plays cardiac hypertrophy by using genetically targeted mouse lines and to identify its potential myocardial substrates via a Shokat approach.

**MATERIALS AND METHODS**

**Mice colonies**

All animal experiments were carried out in accordance with Home Office regulations as detailed in the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986 HMSO (London), which mirrors those of the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH), Bethesda, MD, USA]). We used mice with targeted disruption of p38y and p38 MAPK (p38yKO), the generation of which has been described by Sabio et al. (7). These mice were backcrossed with outbred C57/BL6 mice for at least 5 generations to develop p38y single knockout (KO) mice (p38yKO). PCR protocols for genotyping these mice have been described by Sabio et al. (7).

**Abdominal aortic banding**

Male mice (age 8–10 wk, 22–26 g) from p38yKO or p38yKO colonies were used in all experiments. Mice were subjected to abdominal aortic banding as described by Bogoslavsky et al. (12). In brief, a small metal tube (28 G) was tied against the supra-renal aorta to restrict blood flow and provide increased afterload and a reproducible decrease in renal blood flow to induce cardiac hypertrophy. Buprenorphine (single-dose 20 μg/kg, i.p.; Vetergesic, Ceva Animal Health Ltd, France) was used for perioperative analgesia. After invasive pressure-volume measurements, euthanasia was performed with an overdose of 300 mg/kg pentobarbital (Pentoject). Death was confirmed by monitoring cardiac activity and respiration. For immunohistochemistry, heart excision was performed after terminal anesthesia by intraperitoneal injection of pentobarbital (300 mg/kg) and heparin (150 IU) and flushing of the heart using ice-cold saline.

After excision, hearts were coated in optimum cutting temperature compound (TissueTek; Sakura, Torrance, CA, USA) and snap-frozen in a bath of liquid nitrogen–cooled isopentane and attached to a cork board before cryosectioning at 30 μm.

**Echocardiography and left ventricle function**

All mice received preoperative cardiac ultrasound, and serial echocardiography was used to assess cardiac function after surgery. Mice were anesthetized by using 2% isoflurane (100% oxygen), maintained on a homeothermic platform, and examined by echocardiography using a high-resolution Vevo 770 echocardiography system (VisualSonics, Toronto, ON, Canada) with an RMV-707B transducer at 30 MHz. High-resolution parasternal left ventricle (LV) long- and short-axis M-mode and B-mode images were obtained for offline measurements with Vevo software (VisualSonics) to calculate LV function and mass. At the end of experiments, mice were subjected to invasive LV function assessment by pressure–volume analysis as described by Clark et al. (13).

**Immunocytochemistry**

Mouse cardiac myocytes were isolated as described by Haworth et al. (14). Freshly isolated cardiac myocytes were cultured on laminin-coated culture dishes for 1 h in DMEM (MilliporeSigma, St. Louis, MO, USA). Media were removed and cells were washed with PBS and fixed by 4% paraformaldehyde in PBS for 15 min. This was followed by three 5-min washes with PBS. Cells were then permeabilized with 0.2% Triton X-100 in PBS, followed by a 5-min wash with PBS and blocking with 100 μl/dish of 5% normal goat serum. Specific Abs against pan-isoprotein p38 (9212; Cell Signaling Technology, Danvers, MA, USA), p38β (P38-11A5; Thermo Fisher Scientific, Waltham, MA, USA), p38y (A1347; R&D Systems, Minneapolis, MN, USA), and p38ε (ab188324; Abcam, Cambridge, United Kingdom) were diluted 1:100 in addition to either primary mouse α-actinin (diluted 1:500) or primary rabbit α-actinin (diluted 1:200) in buffer that contained 20 mM Tris base, 155 mM NaCl, 2 mM EGTA, 2 mM MgCl2 pH 7.5, and 1% bovine serum albumin. Dishes were placed into a humid chamber and incubated overnight at 4°C. The next day, cells were washed in PBS and incubated overnight at 4°C with secondary Abs: donkey anti-mouse Cy3 (diluted 1:500; 715-165-150; Jackson ImmunoResearch Labs, West Grove, PA, USA), goat anti-rabbit Alexa Fluor 647 (diluted 1:100; 111-605-003; Jackson ImmunoResearch Labs), and DAPI (diluted 1:100; MilliporeSigma). Cells were then washed as before and mounted with a droplet of mounting medium (0.1 M Tris, 35 ml glycerol, and 2.5 g n-propyl gallate) and covered with 30-mm-diameter glass coverslips (Science Warehouse, Leeds, United Kingdom). The vertical sides of each dish were removed with a hot wire and coverslips were sealed with nail varnish. Each dish was then glued onto a microscope slide and kept at 4°C until analysis by confocal microscopy.

**Immunohistochemistry**

After being cryosectioned and mounted onto poly-lysine slides (Polysine; VWR, Radnor, PA, USA), tissue sections were air dried and briefly fixed with 100% acetone for 15 min at room temperature. Heart sections were circled with wax pen (Dako, Carpinteria, CA, USA), then incubated with PBS that contained 0.1% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin for 20 min. Samples were incubated overnight at 4°C with specific primary Abs against β-myosin heavy chain (β-MHC; diluted 1:200 in β-PBS; MilliporeSigma). The next day, samples were washed with PBS and incubated for an additional 2 h with secondary Ab, diluted 1:200 in PBS and Cy3-conjugated donkey anti-mouse IgG (715-165-150; Jackson ImmunoResearch Labs) and Alexa Fluor 488–conjugated wheat germ agglutinin (Thermo Fisher Scientific). Slides were...
washed and coverslips were placed with mounting medium that contained DAPI (Dako) before being visualized at the appropriate wavelengths by using a Leica SP-5 confocal microscope (Leica Microsystems, Wetzlar, Germany). A total of 5 images from each heart section were used to quantify myocyte cross-sectional area by using a custom macro in ImageJ (v1.50i, NIH), with at least 250 cells being measured for each heart. β-MHC was scored independently as positive (red) cells per high-powered field.

Cloning and purification of recombinant p38γ proteins

The clone of the open reading frame of murine mapk12 (p38γ) was a kind gift from the Rudnicki Laboratory (University of Ottawa, Ottawa, ON, Canada) (15). The full-length insert was subcloned into pETDuet-1 (Thermo Fisher Scientific) 3’ to a 6× His coding sequence. Insertion of cDNA into pETDuet-1 was confirmed by electrophoresis on agarose gels and sequencing. For generating C’ truncated p38γ and AS p38γ, mutations were introduced by using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. For generating AS p38γ, the gatekeeper methionine 109 residue was mutated to a glycine and additional rescue mutations—A160L and L170A—were introduced to prevent the loss of kinase activity. Mutagenesis was confirmed by sequencing. p38γ constructs were transformed into the Escherichia coli-competent strain, Rosetta BL21 (DE3; Bioline, Toronto, ON, Canada). Transformed E. coli were then selected on LB agar medium that contained 100 μg/ml ampicillin. Transformed bacteria were inoculated in LB media that contained 100 μg/ml ampicillin and grown to an optical density of 0.5 at 600 nm, then expression of 6× His protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 5 h at 21°C with constant shaking. p38γ proteins (6× His) were purified by immobilized metal affinity chromatography using nickel-nitriloacetic acid agarose beads (Qiagen, Germantown, MD, USA) and a batch protocol as follows: bacterial cell pellets were resuspended in ice-cold lysis buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 2 mM DTT, and 10 mM imidazole that contained a protease inhibitor cocktail; Roche, Basel, Switzerland). Cell lysis was performed by using a high-intensity ultrasonic processor. Lysates were clarified by centrifugation at 10,000 rpm for 30 min at 4°C. Supernatants were filtered through 0.45-μm membrane filters before being incubated with lysis buffer–equilibrated nickel-nitriloacetic acid agarose beads for 1 h at 4°C on a rotator. Beads were pelleted by centrifugation at 3000 rpm for 5 min at 4°C and the supernatant was discarded. Beads were subsequently washed 6 times with 15 ml ice-cold wash buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 2 mM DTT, and 20 mM imidazole). p38γ proteins (6× His) were eluted by increasing the imidazole concentration stepwise from 0.05 to 0.5 M. p38γ 6× His peak fractions were pooled and dialyzed overnight at 4°C against 2× 2 L of a buffer that consisted of 50 mM Tris, pH 7.4, 100 mM NaCl, and 2 mM DTT. The purity of 6× His p38γ proteins was further refined by ion exchange chromatography on a MonoQ 5/10 column (GE Healthcare Lifesciences, Little Chalfont, United Kingdom) equilibrated in 50 mM Tris, pH 7.4, 100 mM NaCl, and 2 mM DTT. p38γ protein was eluted by applying 40 column volumes of a linear 0.1–0.6 M NaCl gradient. p38γ eluted at ~190 mM NaCl. All chromatographic steps were performed at 4°C. Fractions from each chromatographic step were analyzed by SDS-PAGE and/or Western blot analysis. Fractions that contained highly purified p38γ protein were pooled and used for subsequent biochemical studies. Protein concentration was determined by Pierce Bradford assay (Thermo Fisher Scientific).

Activation of nonactive p38γ proteins

Purified nonactive 6× His p38γ proteins (0.2 mg/ml) were activated by incubation with constitutively active MBP–MAPK kinase 6 (0.02 mg/ml) in the presence of 0.1 mM ATP and 1× kinase buffer (50 mM Tris, pH 7.5, 10 mM MgAc, 0.1 mM EGTA, 0.1% 2-ME) for 1.5 h at 30°C without mixing. To separate MAPK kinase 6 from p38γ proteins, kinase reactions were incubated with amylase resin for 30 min at 4°C. Resin was pelleted by centrifugation at 3000 rpm for 5 min at 4°C, and the supernatant that contained activated protein was collected and analyzed for phosphorylation by SDS-PAGE, Phostag SDS-PAGE, and Western blot analysis. Activated proteins were dialyzed into storage buffer (50 mM Tris, pH 7.5, 0.1 mM EGTA, 270 mM sucrose, 150 mM NaCl, 0.1% 2-ME, 0.2 mM PMSEF, and 1 mM benzamidine) overnight at 4°C.

HEK cell transfection

HEK293 cells were transfected at 70% confluency in Opti-MEM I (Thermo Fisher Scientific) by using Turbofect (Thermo Fisher Scientific) with WT or canonically mutated TAB1 (V408A, M409A; 1-418B; CM) TAB1 plasmid DNA as described by DeNicola et al. (16).

Cloning and purification of recombinant LDB3 protein

His-LDB3 was generated by subcloning the open reading frame of murine ldb3 from the pcR4-TOPO vector (Source Bioscience, Nottingham, United Kingdom) into the pETDuet-1 (Thermo Fisher Scientific) expression vector (Novagen, London, United Kingdom). Subsequent LDB3 cloning and purification steps were as described above for the generation of recombinant p38γ proteins with the following modification: bacteria were cultured at 26°C after IPTG induction.

Cloning and purification of recombinant calpastatin proteins

RNA was extracted from WT mouse heart stored in RNA Later (Thermo Fisher Scientific) by using an RNAsesy Fibrous Tissue Kit (Qiagen) according to the manufacturer’s instructions. cDNA was generated by using Superscript III Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer’s instructions. The resulting RNA/cDNA hybrids were used as templates to generate double-stranded calpastatin DNA using Platinum PfX DNA polymerase (Thermo Fisher Scientific) according to the manufacturer’s instructions. The full-length insert was subcloned into pgEX-3X (GE Healthcare Lifesciences) 3’ to a GST coding sequence. Insertion of cDNA into pgEX-3X was confirmed by electrophoresis on agarose gels and sequencing. To generate calpastatin mutants, mutations were introduced by using the QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer’s instructions. Mutagenesis was confirmed by sequencing. Transformation and expression of WT and mutant calpastatin constructs into E. coli were carried out as described for p38γ with the following variations: 0.1 mM IPTG was used to induce the expression of calpastatin proteins; and bacterial cells were grown at 37°C after induction. GST-calpastatin proteins were purified by glutathione sepharose chromatography. Bacterial cells were resuspended in ice-cold lysis buffer (PBS, pH 7.4, 2 mM DTT, and protease inhibitor cocktail; Roche). Cell lysis was performed by using a high-intensity ultrasonic processor. Lysates were clarified by centrifugation at 4600 rpm for 40 min at 4°C. Supernatant was filtered through a 0.45-μm membrane filter before being incubated with lysis buffer–equilibrated glutathione sepharose beads (GE Healthcare Lifesciences) for 1 h at 4°C on a rotator. Beads were pelleted by centrifugation at 3000 rpm for 1 min at 4°C and the supernatant was discarded. Beads were subsequently washed with 10 ml ice-cold PBS for 2 min at room temperature on a rotator. We carried out 5 additional 10-ml
washes. GST-calpastatin was eluted by applying 1.5-mL volumes of a 10–40 mM reduced glutathione gradient. Fractions that contained GST-calpastatin were pooled and dialyzed overnight at 4°C against 2 × 2 L of buffer that contained 50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT.

**In vitro kinase assays**

Phosphorylation of substrates was performed in vitro in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.2 mM Na$_3$VO$_4$, and 10 mM MgCl$_2$) and contained 200 ng of active p38γ, 1 mM ATP, ATP-γ-S or 6-PhEt-ATP-γ-S (BioLog, Minden, Germany), and 1 μg of recombinant ATF2 (Cell Signaling Technology), α-1-syntrophin (Creative Biomart, Shirley, NY, USA), LDB3, or calpastatin substrate. Reactions were incubated at 30°C for 30 min. Reactions were stopped by adding 2 × Laemmli sample buffer and boiling at 100°C for 5 min. To detect thio-phosphorylated substrates, kinase reactions were stopped with 20 mM EDTA and alkylated with 5 mM p-nitrobenzyl mesylate (PNBM; Abcam) or vehicle for 1–2 h at room temperature before the addition of 2 × Laemmli sample buffer.

**Thiophosphorylation of p38γ substrates in heart homogenate**

Kinase reactions (220 μL) were performed as above but contained 2 mg of heart homogenate, 20 μg of active p38γ, 1 mM Phe-ETH-γ-S, 0.4 mM ATP, and 3 mM GTP. Reactions were incubated at 30°C for 1 h and stopped with 20 mM EDTA. A 10-μL sample of each reaction was removed for alklylation with 5 mM PNM to check the reaction by Western blot analysis using an anti-thiophosphate ester Ab. Samples were frozen in liquid N$_2$ and stored at −80°C until the covalent capture procedure was performed.

**Immunoblot analysis**

Samples were resolved on a 10% reducing SDS polyacrylamide gel and blotted onto a PVDF membrane. Membranes were blocked for 1 h in 1.33% low-fat milk and 0.33% bovine serum albumin in Tris-buffered saline (pH 7.4) and 0.1% Tween-20. Primary Abs were incubated overnight at 4°C with agitation. Abs used included anti–dual-phosphorylated p38 (Thr180 and Tyr182; 4511; Cell Signaling Technology), anti–p38α (1:2000; MA5-15116; Thermo Fisher Scientific), anti–p38β (1:1000; P38-11A5; Thermo Fisher Scientific), anti–p38δ (1:2000; ab188324; Abcam), anti–p38γ (1:8000; AF1347; R&D Systems), anti-phosphorylated ATF2 (1:5000; 9221; Cell Signaling Technology), anti–ATF2 (1:4000; 9226; Cell Signaling Technology), anti–thiophosphatase ester (1:8000; ab92570; Abcam), anti–PDLIM5 (H00100611-A02; Abnova, Taipei, Taiwan), anti–α1-syntrophin (ab11187; Abcam), anti–calpastatin (1:2000; 4146; Cell Signaling Technology), custom anti–dual-phosphorylated calpastatin (T197/S200), and anti–phosphorylated calpastatin (1:1000; 4468; Cambridge Research Biochemicals, Billingham, United Kingdom). After washing and incubating with the appropriate horseradish peroxidase–conjugated secondary Ab (GE Healthcare Lifesciences), Ab–antigen complexes were visualized by ECL (Pierce, Rockford, IL, USA).

**Estimation of p38 MAPK isoform expression**

Whole-heart homogenates were spiked with GST-tagged recombinant proteins (240 ng/well) (Cell Signaling Biotechnology) and probed with isoform-specific mAbs and a total (pan-isoform) Ab as described above. In this way, by using serially diluted recombinant protein (0.4–246.0 mg/well), standard curves were generated for each specific isoform Ab, and the relative endogenous expression of each isoform was determined by using GraphPad Prism (v6; GraphPad Software, La Jolla, CA, USA).

**Phostag SDS-PAGE and silver staining**

For Phostag gel analysis of calpastatin phosphorylation, a Bis-Tris–buffered neutral pH gel system was adopted. For this system, samples were added to 2× lithium dodecyl sulfate loading buffer (282 mM Tris base, 212 mM Tris HCl, pH 8.5, 20% glycerol, 4% lithium dodecyl sulfate, 200 μM DTT, 1 mM EDTA, 0.66 mM SERVA Blue G250, and 0.35 mM Phenol Red) to give a 1× final concentration. Samples were heated to 70°C for 10 min and centrifuged at 10,000 rpm for 2 min. Samples were separated by hard-cast 8% Bis-Tris PAGE mini-gels that were supplemented with 30 μM Phostag acrylamide and 100 μM Zn(NO$_3$)$_2$ with a 4% stacking gel. Gels were electrophoresed at 90 V via the stacking gel for ~20 min and 120 V via the resolving gel. After electrophoresis, gels were stained with silver using a Modified PlusOne Staining Kit (GE Healthcare Lifesciences) according to the manufacturer’s instructions.

**Covalent capture of thiophosphorylated peptides**

Thiophosphorylated peptides were isolated and converted to phosphopeptides for analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described by Dingar et al. (3).

**LC-MS/MS and data analysis**

Analysis of phosphopeptides was carried out by the Bio-Organic Biomedical Mass Spectrometry Resource at University of California, San Francisco (A.L.B.) by using a Linear Trap Quadrupole Orbitrap Velos electron transfer dissociation mass spectrometer (Thermo Fisher Scientific). Peptides were first separated by reverse-phase liquid chromatography using Eksigent nano 1D HPLC (Eksigent, Dublin, CA, USA) that was equipped with a 75 μm × 15 cm reverse-phase C-18 column (LC Packings; Conquer Scientific, San Diego, CA, USA). A 3–32% acetonitrile gradient in 0.1% formic acid was applied (350 nL/min flow rate) to separate peptides. LC-MS/MS was achieved by coupling the reverse-phase column with an atmospheric pressure ionization source in the mass spectrometer. Peptides were analyzed in positive ion mode. The 2 most intense multiple charged peaks from each mass spectrometry spectrum were selected for subsequent fragmentation with both collision-induced dissociation (CID) and electron transfer dissociation mechanisms to generate fragmentation spectra. MS/MS spectra were searched against the UniProt database by using Protein Prospector software to identify proteins and phospho sites.

**LC-MS/MS of calpastatin Phostag**

Excised gel bands were washed in 100 mM NH$_4$HCO$_3$ for 5 min before washing with acetonitrile for 5 min. Supernatant was removed, and gel pieces were rehydrated with additional acetonitrile. Acetonitrile was removed, and gel pieces were dehydrated by using a SpeedVac. Gel pieces were dehydrated by incubating with 10 mM DTT at 56°C for 30 min. Supernatant was removed and 2.5-min acetonitrile washes were repeated as before with subsequent gel dehydration by using a SpeedVac. Next, 55 mM iodoacetamide was added to the samples and incubated at room temperature for 20 min in the dark. Supernatant was discarded, and 2.5-min acetonitrile washes were carried out in a 1:1 ratio.
solution of 100 mM NH₄HCO₃ and acetonitrile at 37°C for 30 min. Supernatant was removed, and 2 5-min acetonitrile washes were carried out with subsequent gel dehydration by using a SpeedVac. Sufficient volume of 13 ng/μl trypsin in 50 mM NH₄HCO₃ to cover gel pieces was added to samples and incubated at 4°C for 20 min. Unabsorbed trypsin was removed, and gel pieces were incubated with a minimal volume of 50 mM NH₄HCO₃ to cover gel pieces. Samples were incubated at 37°C for 2 h, then overnight at room temperature. Gel pieces were washed with 50 mM NH₄HCO₃ at 37°C for 5 min and washed with acetonitrile at 37°C for 10 min. Supernatants from washes were pooled together with a trypsin digest supernatant. Pooled samples were lyophilized by using a speed vacuum. Each sample was resuspended in 1 μl of 50 mM NH₄HCO₃ before LC-MS/MS analysis. Peptides were separated by reverse-phase LC using an EASY NanoLC system (Thermo Fisher Scientific) that was equipped with a 75-μm × 15-cm reverse-phase C-18 column. A 3-step linear gradient of acetonitrile in 0.1% formic acid was applied (300 nl/min flow rate over 180 min) to separate peptides. Eluent was ionized by electrospray ionization using an Orbitrap Velos Pro (Thermo Fisher Scientific) operating with Xcalibur software (v2.2; Thermo Fisher Scientific). Peptides were analyzed in positive ion mode. The instrument was programmed to acquire in automated data-dependent switching mode, selecting precursor ions on the basis of their intensity, for sequencing by CID fragmentation using a Top20 CID method. MS/MS analyses were conducted by using collision energy profiles that were chosen on the basis of the mass-to-charge ratio (m/z) and the charge state of the peptide.

Calpain activity assay

We measured calpain activity in vitro by using purified native calpain I from porcine erythrocytes (EMD Millipore, Billerica, MA, USA) in buffer that contained calcium (50 mM Tris HCl, pH 7.8, 30 mM NaCl, 1 mM DTT, and 3 mM CaCl₂) or buffer without calcium (50 mM Tris HCl, pH 7.8, 30 mM NaCl, 1 mM DTT, and 1 mM EGTA). All assays were in 100 μl total volume in a 96-well format and carried out in triplicate. Each reaction contained 1 μg of calpain I and 20 μM of synthetic Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin fluorescent substrate (Calbiochem, San Diego, CA, USA) in the appropriate buffer. Reactions were incubated for 10 min at 21°C before fluorescence readings were taken using the Gemini XS plate reader (Molecular Devices, San Jose, CA, USA) for 1 h. Fluorophores were excited at 380 nm and the emission of the cleaved substrate was measured at 460 nm.

Mice that lack the p38γ isoform are protected against pressure overload hypertrophy

We observed that homozygous KO mice from the p38γ targeted colony were resistant to cardiac hypertrophy when subjected to supra-renal aortic constriction (banding). WT hearts responded to both stressors by exhibiting a characteristic increase in heart mass (heart mass:body weight and heart weight:tibia length), systolic diameter, and end diastolic volume, as well as a decrease in ejection fraction (Fig. 1A–E). This effect was significantly blunted in p38γKO mice for all relevant parameters. On the basis of the relative abundance of p38γ vs. p38δ (Fig. 1), we hypothesized that this phenotype was the result of p38γ deficiency alone. After backcrossing, mice that lacked only the p38γ isoform demonstrated a response that was similar to those that lacked both p38γ and p38δ. Of note, ejection fraction, fractional shortening, and endocardial fractional area of change were preserved in p38γKO mice at 5 wk after banding (Fig. 3A–C) and KO hearts exhibited significantly less LV chamber dilatation and myocardial wall thickening compared with WT littermates (Fig. 3D–F).

Consequently, hearts from p38γKO mice were significantly smaller than those from WT mice (Fig. 4A), and there were fewer signs of pulmonary edema in KO mice as measured by the postmortem wet: dry weight of the lungs (Fig. 4B). Histochemical analysis of cryopreserved heart tissue revealed that p38γKO hearts had less hypertrophic growth as shown by myocyte cross-sectional area (Fig. 4C) and myocardial expression of the slow isoform of myosin heavy chain (β-MHC) compared with WT littermates, which suggests that remodeling was reduced with the ablation of p38γ (Fig. 4D). We also observed the expression of p38γ in the nuclei of myocytes at 5 wk after banding (Fig. 4E), which is consistent with previous data (17).

RESULTS

Estimation of p38 isoform expression in mouse heart

We first ascertained the relative level of expression of the 4 known p38 isoforms in WT mouse cardiac tissue. When GST-tagged active human recombinant p38β, p38γ, or p38δ MAPK (expected MW, 62 kDa) were added to crude heart homogenates and probed with Abs against each of these isoforms, we observed monoselective binding (Fig. 1A). Data suggest that there was little or no cross-reactivity between isoform-specific Abs. In addition, we used isoform-specific Abs against serially diluted (0.4–246 ng/0.05 mg total protein/well) recombinant p38 MAPK proteins to generate standard curves (data not shown) to determine the relative expression of each isoform in the murine heart (Fig. 1B). Our data suggest that the predominant isoforms that are expressed in the murine heart are p38α and p38γ, which is in agreement with the literature (17). Confocal analysis of isolated mouse cardiac myocytes revealed that p38 MAPK—corresponding mainly to the p38α MAPK isoform—is present in the cytoplasm and the nucleus (Fig. 1C). p38β MAPK is also localized in the cytoplasm and displays a striated pattern (Fig. 1D). p38γ presents a punctuate distribution in the cytoplasm of mouse cardiac myocytes (Fig. 1E) and, of interest, p38δ expression was observed at the intercalated disks and the cytoplasm of cardiac myocytes (Fig. 1F). The selectivity of the signal was confirmed by using cardiac myocytes from p38δKO and p38γ/δKO hearts and a negative control (no primary Ab).

Data analysis

Data are presented as means ± sd. Comparisons between groups were assessed for statistical significance by 1- or 2-way ANOVA or ANOVA with repeated measures, where applicable. Where significance was detected, individual mean values were compared by using Bonferroni’s post hoc test. P values <0.05 were considered statistically significant.

References


To differentiate cardiac substrates of p38γ from other kinase substrates, we set out to identify an N6 expanded ATP analog that could be utilized by AS p38γ, but not by WT p38γ, for phospho transfer. To achieve this, in vitro kinase assays were set up using ATP and 4 different N6 expanded ATP analogs [6-Bn-ATP, 6-PhEt-ATP, 6-Fu-ATP, and 6-(1-MeBu)-ATP]. AS p38γ could utilize all N6 expanded ATP analogs tested to phosphorylate ATF2. In comparison, WT p38γ could not utilize the N6 expanded ATP analogs to phosphorylate ATF2 as efficiently, as a signal for kinase activity.

**Nucleotide specificity of AS p38γ**

Figure 1. Characterization of p38 isoform expression in the mouse heart. Estimation of p38 isoforms in the mammalian heart. A) Whole-heart homogenates were spiked with GST-tagged recombinant proteins (240 ng/well) and probed with isoform-specific mAbs and a total (pan-isoform) Ab. Arrows indicate the endogenously expressed p38α and p38γ also detected in crude heart homogenate. B) After serial dilution of recombinant protein, standard curves were generated for each specific isoform Ab, and the relative endogenous expression of each isoform was determined. C–F) Expression and subcellular distribution of total Ab, corresponding mainly to p38α (C), p38β (D), p38γ (E), and p38δ MAPK (F) isoforms in isolated mouse cardiac myocytes. Negative control (C–E) contained no primary Ab. Data (B) are shown as means ± SD (n = 5/group).
phosphorylated ATF2 was only observed with longer exposures (Fig. 5A, p-ATF2 probe, bottom). From this longer exposure, WT p38γ is less efficient at utilizing 6-PhEt-ATP and 6-(1-MeBu)-ATP than 6-Fu-ATP. 6-PhEt-ATP was chosen for use in all subsequent experiments to label direct substrates of p38γ.

To specifically track and identify substrates of p38γ among the multitude of cardiac proteins, AS p38γ must be able to utilize 6-PhEt-ATPγS, which is further modified at the γ-phosphate to substitute a thiol group to thiophosphorylate downstream substrates (Fig. 5C). This was again assessed in vitro by using ATF2 as substrate (Fig. 5B). To detect thiophosphorylation of ATF2, kinase assays were subsequently alkylated with PNBM. This generates thiophosphate esters that form the epitope for recognition by the thiophosphate Ab. Whereas AS p38γ successfully thiophosphorylated ATF2 by using 6-PhEt-ATPγS, we did not observe thiophosphorylation of ATF2 with WT p38γ or in the nonalkylated AS p38γ control sample.

**Thiophosphorylation and identification of cardiac substrates by AS p38γ**

To identify cardiac substrates of p38γ, we incubated AS p38γ and 6-PhEt-ATPγS with WT murine heart homogenate. Reactions were supplemented with 0.2 mM ATP and 3 mM GTP to reduce background signal caused by use of 6-PhEt-ATPγS by endogenous kinases (Supplemental Fig. 1). Upon termination of assays, a small sample of each assay was removed, alkylated with PNBM, and prepared for immunoblot analysis to monitor the thiophosphorylation reaction (Fig. 5D). Incubation of heart homogenate with both AS p38γ and 6-PhEt-ATPγS results in thiophosphorylation of many cardiac proteins. As thiophosphorylation was successfully determined, the remaining samples were processed in triplicate to enrich phosphopeptides and were analyzed by LC-MS/MS to identify substrates of p38γ. This identified 86 potential cardiac substrates of p38γ (Supplemental Table 1). Among the 86 proteins identified, LDB3 and calpastatin were identified as novel putative substrates of p38γ. LDB3 was determined to undergo phosphorylation at 2 potential sites (Ser98 and Ser240), and calpastatin was determined to undergo phosphorylation at 6 potential sites (Thr216, Ser219, Ser459, Thr460, Ser466, and Thr467). Of interest, both LDB3 sites and 3 calpastatin sites (Thr216, Ser219, and Thr467) are adjacent to a +1 proline residue, which forms the consensus motif for MAPK phosphorylation.
LDB3 and calpastatin are true substrates of p38γ in vitro

To validate LDB3 and calpastatin as true substrates of p38γ, we generated recombinant proteins for use in in vitro kinase assays with p38γ. To overcome the lack of phospho-specific Abs, kinase assays were performed with ATPγS and thiophosphorylation of proteins was examined. WT p38γ successfully thiophosphorylated LDB3 (Fig. 6A) and calpastatin (Fig. 7A). Stoichiometry of the phosphorylation of both substrates by WT p38γ using ATP was examined by Phostag SDS-PAGE gel and silver stain analysis. Phostag SDS-PAGE is a variation of the traditional SDS-PAGE analysis whereby the Phostag reagent captures phosphomonoester di-anions that are bound to serine, threonine, or tyrosine residues and results in a mobility shift of the phosphorylated proteins in SDS-PAGE (16). In both cases, in contrast to control reactions, several higher MW bands were encountered upon LDB3 and calpastatin phosphorylation by p38γ (Figs. 6B and 7B, respectively). This is in agreement with the LC-MS/MS analysis that demonstrated that several sites are targeted for phosphorylation by p38γ. As LDB3 contains a PDZ domain, we investigated whether phosphorylation was PDZ interaction dependent by assessing LDB3 thiophosphorylation using WT and C-terminal truncated p38γ; however, both WT and C′-truncated p38γ phosphorylated LDB3 to the same extent at all the time points measured.

As a positive control, we also examined the thiophosphorylation of α1-syntrophin, a known substrate of p38γ (5). As expected, WT p38γ successfully thiophosphorylated α1-syntrophin (Fig. 6E). TAB1 is a substrate of p38α, and we have previously shown that TAB1 interacts with p38α via 2 distinct sites—at the upper canonical site used by other interacting partners of p38α and a lower, noncanonical TAB1 unique binding site (16). As such, we investigated whether TAB1 is also phosphorylated by p38γ and included reactions with TAB1 mutated at the sites involved in canonical site interaction (CM). We found that, although p38α enhances the phosphorylation of both WT and CM TAB1, as expected, p38γ was not able to phosphorylate TAB1 (Fig. 6D; therefore, TAB1 is not a substrate of p38γ.

We next sought to independently validate the p38γ phoso sites on calpastatin that were previously identified by LC-MS/MS analysis of thiophosphorylated cardiac proteins. For this, we excised bands from Phostag gels and performed LC-MS/MS analysis. This second round of analysis confirmed that all proline-directed sites that were initially detected (Ser98 and Ser240 for LDB3; Thr216, Ser219, and Thr467 for calpastatin) are phosphorylated by p38γ. In addition, several other sites were determined to undergo phosphorylation by p38γ (Supplemental Tables 2 and 3). For subsequent experiments, we focused solely on the p38γ-mediated phosphorylation of calpastatin.

Phosphorylation of Thr216, Ser219, and Thr467 calpastatin by p38γ

Calpastatin contains 4 repetitive inhibitory domains (I–IV) that can each reversibly inhibit 1 calpain molecule (18, 19).
Examination of the peptide sequence and crystal structure of calpastatin revealed that Thr216 and Ser219 are located before the first inhibitory domain of calpastatin, and Thr467 is located between inhibitory domains II and III. Therefore, we engineered double T216A/S219A- and a single T467A phosphorylation-deficient mutants of calpastatin. As before, purified mutants were subject to in vitro kinase assays with p38 and ATP (Fig. 7C). Samples were then probed with custom calpastatin phospho-Abs against these sites. Both phospho-calpastatin Abs—pT216/S216 and pT467—detected phosphorylation of WT calpastatin, but not of the respective alanine mutants, which confirmed that Thr216, Ser219, and Thr467 are phosphorylated by p38 in vitro.

Phosphorylation of calpastatin reduces its inhibitory efficiency

To determine what effect phosphorylation has on the function of calpastatin, we next conducted in vitro fluorometric calpain assays. The synthetic calpain substrate, 7-amino-5-methylcoumarin, is cleaved by calpain, which results in increased fluorescence only when calcium is present in the buffer (Fig. 8A). The addition of recombinant WT calpastatin (0.2–4.5 μg) to the assay in its non-phosphorylated form resulted in the dose-dependent inhibition of calpain activity, as a corresponding decrease in fluorescence was observed (Fig. 8B). Lastly, a significant difference was observed in the inhibition of calpain by...
calpastatin between its nonphosphorylated and p38γ-phosphorylated form (Fig. 8C). Increased fluorescence was observed with phospho-calpastatin, which indicated that phosphorylation of calpastatin by p38γ reduces the inhibitory efficiency of calpastatin. This reduction of the inhibitory effect of calpastatin was reversed when we used a double T216A/S219A phosphorylation–deficient mutant of calpastatin (Fig. 8C).

**DISCUSSION**

The purpose of the current study was to decipher the role played by p38γ in the heart. To summarize our findings, we have shown that p38γ is expressed in the heart and is an important regulator of the progression of cardiac hypertrophy. By using the Shokat approach to identify substrates of protein kinases, we have discovered LDB3 and calpastatin, among other substrates, to be novel targets of p38γ. Both proteins are indispensable to cardiac myocyte function. LDB3 is a key cytoskeletal protein in cardiomyocytes, and calpastatin is the natural and endogenous inhibitor of calpain proteases. Although we were unable to assess the consequences of p38γ-mediated LDB3 phosphorylation, we observed that phosphorylation of calpastatin by p38γ reduces the efficiency of calpastatin in inhibiting calpain. If this disinhibition occurs in vivo, it may be an important mechanism by which p38γ mediates its prohypertrophic role in the heart.

**Phenotype of p38γKO mice after cardiac stress**

Data presented here confirm the prohypertrophic role of p38γ in the myocardium. Global p38γKO mice are less susceptible to the effects of pressure overload hypertrophy compared with WT controls (Figs. 2 and 3), which is consistent with the recent findings by the Sabio group (9). The LV of WT mice gradually increased in mass and contraction was impaired, as indicated by an increase in internal diameter. Despite the roles for this isoform that have been described in skeletal muscle development and maintenance, blunted growth of p38γKO mice was not a confounding factor in this study (20).

Immunohistochemistry of hearts revealed an increase in the expression of β-MHC and an increase in myocyte
cross-sectional area, both of which are hallmarks of experimental cardiac hypertrophy (5, 21, 22), in banded WT but not KO hearts (Fig. 3), which supports the observation that p38\(\gamma\) is involved in the progression of pathologic cardiac hypertrophy.

**Cardiac substrates of p38\(\gamma\)**

Two novel cardiac substrates of p38\(\gamma\) identified in the current study are LDB3 and calpastatin. Extensive characterization of these proteins and their p38\(\gamma\) phosphorylation sites has been carried out in vitro, and we have confirmed the phosphorylation of both proteins by p38\(\gamma\) (Figs. 6 and 7). Phastag gel analysis, mass spectrometry, and custom phospho-Abs against the phosphorylated protein substrates demonstrate that both substrates are phosphorylated by p38\(\gamma\) at multiple sites. LDB3 is phosphorylated by p38\(\gamma\) at Ser\(^98\) and Ser\(^240\), and calpastatin is phosphorylated by p38\(\gamma\) at Thr\(^197\)/Ser\(^200\) and Thr\(^448\) (Fig. 7). All sites, to our knowledge, are novel phosphorylation sites that have not previously been described. Of interest, both substrates identified here have previously been implicated in cardiac hypertrophy, as discussed further below.

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**Figure 6.** *In vitro* validation of LDB3 and α1-syntrophin, but not TAB1, as substrates of p38\(\gamma\). A) Immunoblot analysis of LDB3 (1 μg) thio-phosphorylation by WT p38\(\gamma\) (200 ng) using ATP\(^{γ}\)S (1 mM) after 30 min at 30°C. B) Phastag SDS-PAGE gel and silver stain analysis of LDB3 phosphorylation by WT p38\(\gamma\) with ATP (1 mM) after 30 min at 30°C (dotted line indicates where irrelevant lanes have been removed for clarity). C) Immunoblot analysis of LDB3 (1 μg) thio-phosphorylation by WT or C\(\varepsilon\)-truncated p38\(\gamma\) (200 ng) using ATP\(^{γ}\)S after 1, 5, or 10 min. D) Immunoblot analysis of WT TAB1 or CM TAB1 phosphorylation by WT p38\(\alpha\) and p38\(\gamma\) with ATP after 30 min at 30°C. E) Immunoblot analysis of α1-syntrophin (1 μg) thio-phosphorylation by WT p38\(\gamma\) using ATP\(^{γ}\)S after 30 min at 30°C.
**LDB3**

LDB3 is a key cytoskeletal scaffold protein in cardiomyocytes (23). LDB3-null mice are not viable, and cardiac-specific or inducible cardiac-specific LDB3 KO mice display disrupted cardiomyocyte structure, decreased cardiac function, and eventually develop a severe form of dilated cardiomyopathy, followed by premature death (24). Furthermore, mutations in the ZASP gene (human ortholog) have been associated with dilated cardiomyopathies (25) and LV noncompaction (25, 26) in humans, which demonstrates the essential role of LDB3 in the heart.

Of interest, LDB3 contains a PDZ domain; however, the interaction of the PDZ domain of LDB3 with the PDZ domain-interacting motif of p38γ was not necessary for successful substrate phosphorylation, as C' truncated p38γ could phosphorylate LDB3 to the same extent as WT p38γ, at least under *in vitro* experimental conditions tested here (Fig. 6).

**Calpastatin**

Calpastatin is the natural and endogenous inhibitor of calpain proteases. Calpain proteases are cysteine-dependent proteases that are activated by increased intracellular calcium in such scenarios as cardiac hypertrophy and remodeling. Previous studies have demonstrated that inhibition of calpain activity during pathophysiologic circumstances demonstrates favorable improvements in cardiac function (27–29). Results from animal studies of calpain in cardiac remodeling are supported by data from patients with congestive heart failure. A significant increase in calpain 1 and 2 protein and a concomitant increase in the cleavage of calpain substrate cabin 1 has been reported in severe failing hearts compared with hearts from healthy controls (30).

In support of previous studies (31, 32), we have clearly demonstrated that phospho modification of calpastatin can modulate its ability to inhibit calpain. We speculate that this may be a mechanism by which p38γ mediates its prohypertrophic role in the heart. A crucial substrate of calpain is the serine/threonine-specific phosphatase, calcineurin. Elevation in intracellular calcium or cleavage of the autoinhibitory domain of calcineurin by calpain can activate calcineurin (33). Once active, calcineurin dephosphorylates nuclear factor of activated T cell (NFAT) transcription factors, key prohypertrophic transcription factors in the heart. Dephosphorylated NFAT transcription factors then translocate to the nucleus to increase transcription of prohypertrophic genes. In principle, this could place p38γ within a well-established hypertrophic signaling cascade. p38γ-mediated phosphorylation of calpastatin would result in unrestrained calpain activity, increased calcineurin activation, and a concomitant increase in NFAT activity. Although other targets of p38γ may contribute to the development of pathologic cardiac hypertrophy, this pathway may explain, in part, the resistance of p38γKO mice to...
pressure overload hypertrophy compared with WT mice and warrants additional investigation.

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AUTHOR CONTRIBUTIONS

A. A. Loonat, M. S. Marber, and J. E. Clark designed research; A. A. Loonat, N. Sarafraz-Shakary, K. Tilgner, S. Uddin, M. Thomas, and J. E. Clark performed in vivo experiments and analyzed data; A. A. Loonat and E. D. Martin performed in vitro research; A. A. Loonat, M. S. Marber, and J. E. Clark wrote the manuscript; and N. T. Hertz, R. Levin, K. M. Shokat, and A. L. Burlingame contributed new reagents or analytic tools.

REFERENCES


Figure 8. Phosphorylation of calpastatin by p38 MAPK reduces its ability to inhibit calpain in vitro. Cleavage of the synthetic calpain substrate, Suc-Leu-Leu-Val-Try-AMC (amino-4-methylcoumarin; 20 μM), by calpain (1 μg) liberates the AMC fluorophore. A) Fluorescence or calpain activity is only observed in the presence of 1 mM calcium. B) Dose-dependent inhibition of calpain by calpastatin (0.2–4.5 μg). C) Calpain activity (normalized to percentage of maximal activity) in the presence of WT calpastatin (1.8 μg), phosphorylated calpastatin, and T216A/S219A phosphor-dead mutant calpastatin. Data are shown as means ± SEM (n = 3). *P < 0.05 vs. calpain activity positive control, †P < 0.05 vs. calpain activity with nonphosphorylated calpastatin, ‡P < 0.05 vs. phosphorylated WT calpastatin.