Acetylation of a Conserved Lysine Residue in the ATP Binding Pocket of p38 Augments Its Kinase Activity during Hypertrophy of Cardiomyocytes[▽]

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Like phosphorylation, acetylation of lysine residues within a protein is considered a biologically relevant modification that controls the activity of target proteins. During stress of cells, massive protein acetylation takes place. Here, we show that p38 mitogen-activated protein kinase (MAPK), which controls many biological functions during stress, is reversibly acetylated by PCAF/p300 and HDAC3. We identified two acetylated lysine residues, K152 and K53, located in the substrate binding domain and in the ATP-binding pocket of p38, respectively. Acetylation of lysine 53 enhanced the activity of p38 by increasing its affinity for ATP binding. The enhanced acetylation and activation of p38 were found to be in parallel with reduced intracellular ATP levels in cardiomyocytes under stress, as well as *in vivo* models of cardiac hypertrophy. Thus, our data show, for the first time, that p38 activity is critically regulated by, in addition to phosphorylation, reversible acetylation of a lysine residue, which is conserved in other kinases, implying the possibility of a similar mechanism regulating their activity.

Throughout life, cells in our body are subjected to constant damage and stress. A variety of extracellular stimuli are converted into specific cellular responses through activation of mitogen-activated protein kinase (MAPK) signaling pathways. p38 belongs to stress-activated MAPKs that are activated upon phosphorylation. Activation of p38 has been seen in response to a variety of environmental stressors, such as UV light, heat shock, osmotic stress, inflammatory cytokines (interleukin 1 [IL-1] and tumor necrosis factor alpha [TNF- α]), and growth factor stimulation (13, 19, 23, 35, 45). The downstream targets of p38 include several transcription factors and components of the translational machinery (37, 42, 55, 59). Thus, this kinase is capable of regulating many diverse biological processes, including cell growth/differentiation, cell cycle arrest, apoptosis, cardiomyocyte hypertrophy, inflammation, senescence, and tumor progression (4, 20, 22, 36, 39, 51).

In regard to mechanisms regulating p38 activity, two mechanisms have been proposed so far. The first is protein phosphorylation by dual kinases, termed MAP kinase kinase (MKK), specifically MKK3 and MKK6 (27); the second is the interaction of p38 with TAB1, leading to autophosphorylation of the enzyme (11). p38 has been also shown to be modified by nitration, leading to reduced activity of the kinase (56). However, another posttranslational modification which regulates p38 activity has not been identified yet.

Severe oxidative stress has been shown to deplete intracel-

lular ATP levels due to hemichannel-mediated loss of ATP and/or by enhanced consumption of ATP to meet the increased metabolic demand of the cell (1, 12). In the heart, mechanical stretch and oxidative stress of cardiomyocytes have been implicated in the induction and progression of cardiac hypertrophy (26, 49). Reactive oxygen species generated during oxidative stress have been shown to activate a variety of stress-activated kinases, including p38MAPK (hereafter p38), which are involved in the activation of cardiac hypertrophic signaling (52). Of the four isoforms of p38 known, p38 α is the major isoform expressed in adult hearts and is activated during stress of cardiomyocytes (40). The activation of p38 α has been shown to induce phosphorylation of nuclear and transcriptional regulatory proteins, such as NFAT, GATA4, c-Jun, p53, ATF-2, Elk-1, MEF-2, Max, and MAPK-activated protein kinase 2/3 (MAPK-APK2/3) (30). The overactivation of these factors has been shown to be associated with the development of cardiac hypertrophy. p38 activation in dilated and end-stage failing human hearts has been also reported (8, 21). We and others have shown that cardiac hypertrophy is also associated with depletion of ATP (15, 41), suggesting that ATP-dependent proteins, like MAPKs, which are involved in combating stress need to maintain their activity under energy-depleted conditions, underlining the potential need for additional posttranslational modifications that would enable proteins to work efficiently in energy-depleted conditions.

Protein acetylation at lysine residues is emerging as an important posttranslational modification that regulates the activity of the target protein. Lysine acetylation is a reversible posttranslational modification process in which histone acetyltransferases (HATs) transfer the acetyl moiety from acetyl coenzyme A (CoA) to the [element]-amino groups of lysine

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(K) within a protein, resulting in elimination of its positive charge. The opposite of acetylation is carried out by another group of enzymes called histone deacetylases (HDACs), which are classified into three main classes based on sequence homology and their dependency on the cofactor (25). Reversible acetylation of a lysine residue might cooperate with or rival phosphorylation to regulate activity of the target protein. Recent evidence suggests that during stress of cells, many non-histone proteins are acetylated (3). However, acetylation of p38, which is a major mediator of the stress response of cells, has never been investigated. In this study, we report that p38 is an acetylated protein and acetylation of p38 at K53 of the ATP-binding pocket enhances p38 activity during cellular stress.

MATERIALS AND METHODS

Antibodies used. The following antibodies and conjugates were used in this study: rabbit anti-p38 (no. 9218; Cell Signaling), mouse anti-phospho (Pho)-p38 (9216; Cell Signaling), mouse anti-p38 (sc-7972; Santa Cruz), mouse anti-glutathione *S*-transferase (anti-GST) (sc-132; Santa Cruz), rabbit anti-Flag (ab1162; Abcam), rabbit antihemagglutinin (anti-HA) (sc-805; Santa Cruz), rabbit anti-phospho-ATF2 (9225; Cell Signaling), rabbit anti-JNK1 (9252; Cell Signaling), mouse anti-phospho-JNK/SAP (9251; cell signaling), rabbit anti-PCAF (sc369; Santa Cruz), goat anti-HDAC3 (sc8183; Santa Cruz), goat anti-Akt1 (sc-1618; Santa Cruz), rabbit anti-phospho-Akt (4060; Cell Signaling), rabbit anti-extracellular signal-regulated kinase 1 (anti-ERK1) (Santa Cruz), rabbit anti-phospho-ERK1/2 (4370; Cell Signaling), rabbit anti-green fluorescent protein (anti-GFP) (Sc-8334; Santa Cruz), mouse anti-acetylated-lysine (anti-Ac-K-103) (9681; Cell Signaling), and rabbit acetylated-lysine rabbit anti-acetylated-lysine (06-933; Upstate).

Plasmid constructs. The constructs HA-p38 alpha (Addgene; plasmid 12658 deposited by John Kyriakis), HDAC1 (Addgene; plasmid 13820 deposited by Eric Verdin), HDAC3 (Addgene; plasmid 13819 deposited by Eric Verdin), p300 (Addgene; plasmid 10717 deposited by William Sellers), PCAF (Addgene; plasmid 8941 deposited by Yoshihiro Nakatani), and MKK3 (Addgene; plasmid 12186 deposited by Gary Johnson) were used. HA-p38 mutants were made by site-directed mutagenesis. For GST plasmids, p38 was amplified from HA-p38 with primers flanking the BamHI and XhoI restriction enzyme sites and cloned into pGEX-KG vector. Different GST mutants were made by site-directed mutagenesis of this vector. Histidine (His)-p38 was made by PCR, amplifying p38 from HA-p38 using primers flanking the BamHI and PstI restriction enzyme sites and cloning it in the pQE-80 vector. K-to-R His-p38 mutants were generated by PCR-based site-directed mutagenesis. Plasmids for short hairpin RNA (shRNA) HDAC3, pBS/U6-HDAC3, and empty pBS/U6 have been described previously (58).

RNA interference analysis. HeLa cells were transfected with 100 nM ON-TARGETplus small interfering RNA (siRNA) specific for human PCAF or p38 using DharmaFect transfection reagents per the manufacturer's instructions. After 72 h of transfection, cells were harvested, and lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer. p38 immunoprecipitated (IP) from these lysates was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then analyzed by immunoblotting (IB).

Coimmunoprecipitation assay. To analyze the interaction of different HDACs with p38, expression plasmids for Flag-tagged HDACs were transfected into HeLa cells along with a construct expressing HA-p38. For analysis of interaction between p300 (or PCAF) with p38, an expression plasmid for HA-p38 was transfected into HeLa cells along with a construct expressing Flag-PCAF or p300. After 48 h of transfection, cells were washed twice with phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton X-100, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM Na₂EDTA, 1 mM EGTA, 1 mM beta-glycerophosphate, 1 mM sodium *ortho*-vanadate, 1 mM sodium fluoride, and 10 µl/ml protease inhibitor [Sigma]). Bound proteins were IP with Flag beads (Sigma), separated by SDS-PAGE, and detected by immunoblotting with anti-HA (Santa Cruz) and anti-Flag (Sigma) antibodies. Five percent milk in Tris-buffered saline (TBST) with 0.1% Tween 20 was used for membrane blocking, and 1% milk in TBST with 0.1% Tween 20 was used for antibody incubation. Blots were developed with a chemiluminescent kit (Pierce). For analysis of *in vivo* acetylation, cardiomyocytes or HeLa cells were washed twice with PBS and lysed in buffer K (20 mM

Tris-HCl [pH 7.5], 150 NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM PMSF, and 30 µM trichostatin A [TSA]). Soluble extracts were prepared for immunoprecipitation with anti-p38 antibody and immunoblotted with anti-pan-acetyl-lysine (anti-Ac-K) antibody. Anti-p38 antibody detects p38α MAPK

Protein-protein interaction *in vitro*. For *in vitro* protein binding assays, 2 µg of His-p38 was captured on nickel-nitrilotriacetic acid (Ni-NTA) beads. Briefly, the plasmid pQE-his-p38 was expressed in bacteria, and cells were lysed in lysis buffer, containing 50 mM Tris HCl (pH 8), 250 mM NaCl, 10 mM imidazole, and protease inhibitor cocktail (Sigma). The cleared supernatant was agitated with Ni-NTA resin for 1 h at 4°C. The resin was washed 3 times with the lysis buffer and then washed 3 times with 1× HAT buffer. GST-tagged p300 or PCAF (Upstate Biotechnology) was incubated with His-p38 on Ni-NTA beads and incubated in acetylation buffer with 0.5 mM acetyl-CoA for 1 h at 30°C. Beads were washed 5 times with wash buffer, separated by 10% SDS-PAGE, and probed with anti-GST or anti-pan-acetyl-lysine antibodies.

***In vitro* acetylation assay.** Histidine-tagged phospho-p38 (R&D Systems) (60 ng) was incubated with 1 µg active PCAF enzyme (Upstate Biotechnology), 0.5 mM acetyl-CoA (Sigma), 50 mM nicotinamide (Sigma), and 50 µM TSA in 1× HAT buffer (50 mM Tris [pH 8], 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol [DTT]) for 1 h at 30°C. Unacetylated p38 contains all these components except acetyl-CoA.

Deacetylation assay. Purified histidine-tagged p38 caught on Ni-NTA beads was subjected to *in vitro* acetylation using GST-PCAF as described above. Acetylated His-p38 beads were washed as described above and resuspended in 1× HDAC buffer (50 mM Tris [pH 8.0], 4 mM MgCl₂, 0.2 mM DTT). Acetylated p38 was incubated either in just 1× HDAC buffer (control) or with Flag-tagged HDAC3, HDAC1, or HDAC5 and immunoprecipitated from HeLa cells. Reaction mixtures were incubated for 2 to 3 h at 37°C on a rotator. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

GST protein purification. Transformants were grown at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.6 and induced using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The resulting fusion proteins were purified according to the manufacturer's protocol by binding them to glutathione-Sepharose 4BTM (Amersham).

***In vitro* ATP binding assay.** Histidine-tagged phospho-p38 bound to Ni-NTA beads was subjected to *in vitro* acetylation as described above. The control was subjected to similar treatment in the absence of acetyl-CoA. Beads with proteins were washed 3 times with 50 mM Tris (pH 7.5). Beads with proteins were incubated in 50 µl of Tris (pH 7.5) with ³²P-labeled ATP at 30°C for 30 min. Beads were washed 5 times with 50 mM Tris (pH 7.5) and subjected to scintillation counting. p38 was preincubated with 5 µM SB203580 in 40 µl of 50 mM Tris (pH 7.5) for 30 min followed by incubation with [³²P]ATP at 30° for 30 min as indicated. Beads were separated and washed twice with PBS, and the radioactivity associated with beads was measured by the use of a scintillation counter.

***In vitro* kinase assay.** *In vitro* kinase assays for p38 MAPK and JNK1 were performed using p38 MAPK kinase assay kit from Cell Signaling. Briefly, kinase reactions were carried out in the kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂ supplemented with 200 µM ATP or as indicated, and 1 µg glutathione *S*-transferase-activating transcription factor 2 (GST-ATF2) fusion protein as a substrate at 30°C for 30 min. Reactions were stopped by adding a 2× SDS-PAGE sample buffer, and the samples were run on a 10% SDS-PAGE gel and immunoblotted. Incorporation of the phosphorus residue into the GST-ATF2 protein was detected with the phospho-specific ATF2 (Thr71) antibody (Cell Signaling).

Annexin V apoptosis assay. HeLa Cells were cultured in 6-well plates at 70% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum albumin (FBS) and were treated with 500 ng/ml of anisomycin for 4 h in serum-free medium. In the set of experiments in which p38 was transfected, anisomycin treatment was given only for 2 h. Assays were performed according to the manufacturer's protocol (BD Biosciences); briefly, all cells were harvested from tissue culture plates and centrifuged at 1,500 rpm for 5 min at 4°C. Supernatant was removed, and cells were washed twice with cold PBS. Cells were then resuspended in 100 µl of cold 1× binding buffer, and 5 µl of annexin V-PE was added. Samples were incubated for 15 min in the dark at room temperature. A total of 400 µl of 1× binding buffer was added and analyzed by flow cytometry using a FacScan analyzer (Becton-Dickinson, San Jose, CA). Results were processed using FlowJo software.

Cell culture and transfection. HeLa cells or 293T cells were maintained in DMEM with 10% fetal bovine serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin in a 37°C incubator with 5% CO₂. Primary cultures of 2-day-old neonatal rat heart myocytes were carried out using an established protocol as

described previously (50). All animal protocols were reviewed and approved by the University of Chicago Animal Care and Use Committee. For transfections, 1.2×10^6 cells were grown in 10-cm plates and were transfected with appropriate plasmids using Superfect transfection reagent (Qiagen) according to the manufacturer's protocol. shRNA plasmid transfections were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and cells were harvested 72 h after transfections in RIPA buffer.

Cardiomyocyte culture and mechanical stretch. Primary cultures of 2-day-old neonatal rat heart myocytes were carried out using an established procedure described previously (43). Cells were seeded at a concentration of 0.5×10^6 /ml on Bioflex plates and subjected to cyclic mechanical stretch. The frequency of cyclic stretch was 0.2 Hz, with a pulsation of 10% elongation 12 times/minute.

Induction of hypertrophy in mice. Isoproterenol dissolved in 150 mM NaCl and 1 mM acetic acid was delivered chronically at a rate of 8.7 mg/kg of body weight/day for 7 days by implanting osmotic minipumps (Alzet model 2002) in the peritoneal cavity of mice. Control mice underwent the same procedure, except that the respective pumps were filled only with vehicle (150 mM NaCl, 1 mM acetic acid).

ATP estimation. ATP levels were measured using a bioluminescence assay kit (Roche Applied Science). Cells (1×10^5) or frozen crushed tissue (50 mg) was lysed in 200 μ l of cell lysis reagent (Roche Applied Science). Luciferase reagent (100 μ l) and dilution buffer (50 μ l) were added to 50 μ l of lysate, and the luminescence was analyzed after a 1-s delay with 10-s integration on a luminometer. A standard curve was generated from known amounts of ATP and used to calculate the ATP content of the sample. Values were normalized to the total protein content of the sample.

Mass spectrometry. Protein was resolved by SDS-PAGE, and the band to be analyzed was excised from the gel by the use of a razor blade and divided into ~ 1 -mm³ pieces. Gel pieces were destained using 100 mM ammonium bicarbonate (pH 8.9) in 50% acetonitrile and then treated with 100 μ l of 50 mM ammonium bicarbonate (pH 8.0) and 10 μ l of 10 mM TCEP [Tris(2-carboxyethyl)phosphine HCl] at 37°C for 30 min. Protein digestion was carried out using 1:50 sequencing-grade trypsin or chymotrypsin in 50 mM ammonium bicarbonate (pH 7.5). Digested peptide samples were desalted with a C₈ OptiPak column (Optimize Technologies) and analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI/MS/MS) on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer. Positive-ion mass spectra were acquired in the reflectron mode. Ions selected for MS/MS were subsequently placed on an exclusion list using an isolation width of 1.6 Da, a low-mass exclusion of 0.8 Da, and a high-mass exclusion of 0.8 Da. Tandem mass spectra were extracted by Readw.exe version 3.0. All MS/MS samples were analyzed using Mascot (Matrix Science, London, United Kingdom) data explorer software, and Scaffold (version Scaffold_2.1.03; Proteome Software, Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications.

Statistical analysis. Student's *t* test was used to analyze statistical significance between two groups. All *P* values corresponded to two-tailed tests, and a *P* value of <0.05 was considered statistically significant.

RESULTS

Stress induces p38 acetylation. Previous studies have led to the identification of a profound increase in the acetylation of several proteins after a mechanical stretch of cardiomyocytes (43). Because cell stress is known to activate the MAPK pathway, we tested the phosphorylation status of different kinases of this pathway in mechanically stretched cardiomyocytes. We found that, while the activity of Pho-p38 and Pho-JNK was considerably elevated, the activity of Pho-ERK remained unchanged and Pho-AKT was reduced (Fig. 1A). One of the protein bands which were acetylated during the stretch of myocytes was around 42 kDa in molecular mass. We presumed that this band could be p38. To confirm this observation, we immunoprecipitated p38 from cells subjected to mechanical stretch or treated with angiotensin-II, a stress-inducing agent, and tested for acetylation of p38. In both stress situations, p38 was highly acetylated (Fig. 1B and C). These results thus indicated that p38 was acetylated as well as phosphorylated during stress of cardiomyocytes.

We asked then whether p38 can be acetylated in nonmuscle cells. For this purpose, we treated HeLa cells with trichostatin A (TSA), a class I and II HDAC inhibitor. The cell lysate was IP with p38 antibody, and the resulting complex was analyzed by IB, using an anti-acetyl-lysine (anti-Ac-K) antibody. We found a notable increase in the acetylation of p38 in TSA-treated samples, indicating that the inhibition of class I and II HDACs resulted in acetylation of p38 (Fig. 1D). Because ATP depletion is associated with cellular stress, we next examined ATP levels in cells in which p38 was acetylated. In all three conditions (mechanical stretch, angiotensin-II, and TSA treatment) under which p38 was acetylated, there was a significantly reduced intracellular level of ATP (Fig. 1E, F, and G), highlighting that p38 acetylation is associated with reduced cellular ATP levels.

PCAF and p300 are capable of acetylating p38. PCAF and p300 are two common mammalian HATs which are known to acetylate target proteins. To determine which member of the HAT family binds to p38, we expressed His-tagged p38 (p38 α) in bacteria and purified the kinase with Ni-NTA agarose beads. Beads containing p38 were incubated with GST, GST-PCAF, or GST-p300 (HAT domain) in an acetylation buffer containing acetyl-CoA. After completion of the reaction, agarose beads were separated and analyzed by IB. HAT enzymes incubated with beads containing the His tag alone were used as negative controls. We found that p38 was able to pull down GST-PCAF and GST-p300 under *in vitro* assay conditions. When the same blot was probed with anti-Ac-K antibody, we realized that HATs binding to p38 were also capable of acetylating the kinase (Fig. 2A). We then examined this interaction *in vivo*. For this purpose, cells were overexpressed with two epitope-tagged proteins, HA-p38 and Flag-PCAF. Lysate of these cells was subjected to IP with anti-Flag or anti-HA antibodies, and the resulting beads were analyzed by IB with the use of antibodies against the protein which was sought to be coprecipitated. HA-p38 was coprecipitated with Flag-PCAF (Fig. 2B). This was also confirmed by reverse IP, in which Flag-PCAF was found to be bound with HA-p38 (Fig. 2C). To examine whether p38 was acetylated by PCAF *in vivo*, the lysate of cells, expressed with HA-p38 in combination with Flag-PCAF or catalytically mutant PCAF, was IP with anti-HA antibody, and the resulting beads were subjected to IB with anti-Ac-K antibody. The results showed that p38 was highly acetylated by wild-type PCAF and not by catalytically inactive mutant PCAF *in vivo* (Fig. 2D). Similarly p38 IP from PCAF knockdown HeLa cells showed decreased acetylation (Fig. 2E and F). To confirm the interaction of endogenous p38 with PCAF, HeLa cell lysate was subjected to IP with anti-p38 antibody or anti-HA antibody, and the resulting complex was analyzed by IB with antibodies against PCAF and p38. As shown in Fig. 2G, PCAF was specifically pulled down by anti-p38 and not by anti-HA antibody. Similar experiments were carried out with p38 and p300, and identical results were obtained. These results thus indicated that PCAF and p300 were capable of binding to p38 and acetylating it *in vivo*.

HDAC3 interacts with and deacetylates p38. The effect of HATs is counteracted by HDACs. To delineate the HDAC associated with p38, we first examined interaction of p38 with different members of class I and II HDACs. HeLa cells were overexpressed with HA-p38 together with Flag-HDAC1,

FIG. 1. Cardiomyocyte stress induces p38 acetylation. (A) Expression levels of phospho (Pho)-p38, Pho-JNK, Pho-ERK, and Pho-AKT after mechanical stretching (MS) of cardiomyocytes. (B, C) Cardiomyocytes were subjected to 10% MS for 4 h or treated with 1 μ M angiotensin-II for 24 h. Cell lysate was subjected to immunoprecipitation (IP) with an anti-p38 antibody. The resulting immune complex was analyzed by IB with antibodies against acetyl lysines (Ac-K) and p38. Arrow points to acetylated p38 band. The sizes of the molecular weight markers are indicated on the right. (D) HeLa cells were treated overnight with 500 ng of TSA. Cell lysate was subjected to IP with p38 antibody, and the complex was analyzed by IB using anti-Ac-K and anti-p38 antibodies. (E, F) Quantification of ATP levels in MS and Ang-II treated cardiomyocytes. (G) ATP levels in HeLa cells treated with TSA. C, control. Results shown are mean $\pm$ standard error (SE). $n = 4$.

-HDAC3, or -HDAC5. We found that Flag-HDAC3 was co-precipitated with HA-p38, but not other HDACs (Fig. 3A). This interaction was also confirmed by reverse IP, in which HA-p38 was seen coprecipitated with Flag-HDAC3 (Fig. 3B). To confirm the interaction of endogenous p38 with HDAC3, HeLa cell lysate was subjected to IP with anti-p38 antibody or anti-HA antibody, and the resulting complex was analyzed by IB with antibodies against HDAC3. HDAC3 was specifically pulled down by anti-p38 and not by anti-HA antibody (Fig. 3C), thus demonstrating that HDAC3 is capable of binding to p38 *in vivo*.

We tested next the ability of HDAC3 to deacetylate p38. HeLa cells were overexpressed with HA-p38 together with Flag-HDAC1, -HDAC3, or -HDAC5. HA-p38 was subjected to IP from cell lysate and was analyzed by IB with the use of anti-Ac-K and anti-HA antibodies. The results demonstrated that p38 acetylation was notably reduced in cells expressed

with Flag-HDAC3, but not with other HDACs (Fig. 3D). To further confirm these results, we examined the ability of HDAC3 to deacetylate p38 *in vitro*. These beads containing Flag-HDAC3, Flag-HDAC1, or Flag-HDAC5 were incubated with *in vitro* acetylated-p38 in a deacetylation buffer. The results indicated that HDAC3, but not HDAC1 or HDAC5, had the ability to deacetylate p38, thus confirming that p38 is a target of HDAC3-mediated deacetylation (Fig. 3E).

Acetylation enhances p38 activity by increasing its affinity for ATP. Having shown that p38 is reversibly acetylated *in vivo* and p38 acetylation is associated with reduced ATP level in cells, we investigated next the impact of acetylation on the activity of p38. For this, we subjected phospho-p38 to acetylation with PCAF and then tested for its ability to phosphorylate ATF2, a *bona fide* substrate of p38 (ATF2 used in this assay is recombinant ATF2, 19 to 96 amino acids fused to GST) at gradually increasing time points of the kinase reaction. As

FIG. 2. p38 is acetylated by PCAF/p300. (A) Histidine-tagged p38 on beads was subjected to *in vitro* acetylation using GST-PCAF or GST-p300. Beads were separated, washed, and subjected to SDS-PAGE and IB with indicated antibodies. The sizes of the molecular weight markers are indicated on the left. (B to D) HeLa cells were overexpressed with indicated plasmids. Cell lysate was subjected to IP with indicated antibodies, and the resulting beads were analyzed by IB using antibodies shown on the right. (E) HeLa cells were subjected to PCAF knockdown by use of scrambled or PCAF-specific siRNA. Cell lysates were subjected to IP with anti-p38 or anti-HA antibodies. The resulting beads were analyzed by IB with the use of indicated antibodies. (F) IB analysis of whole-cell lysate (WCL) showing PCAF knockdown in scrambled or PCAF-specific siRNA-treated cells. (G) HeLa cell lysate was subjected to IP with anti-p38 or anti-HA antibodies, and the resulting beads were analyzed by IB with the use of indicated antibodies.

shown in Fig. 4A, acetylated p38 showed increased phosphorylation of ATF2, suggesting that acetylated p38 had a significantly increased rate of enzymatic activity compared to its nonacetylated counterpart. The activity of p38 can be blocked by the inhibitor SB203580 (SB), which binds to the ATP-binding pocket of p38 and interferes with its ATP-binding ability (22). To test whether acetylation could have augmented the activity of enzyme by enhancing its affinity for ATP, we incubated [32 P]ATP at different concentrations with acetylated, nonacetylated, or SB inhibitor-bound p38 in a Tris buffer. The results showed that acetylated p38 had significantly higher ATP binding ability than the nonacetylated control (Fig. 4B). The use of SB inhibitor completely abolished the ATP binding ability of p38 as expected (Fig. 4B).

To confirm further that p38 activity after acetylation was enhanced by its increased ATP-binding ability, we subjected ATF2 to phosphorylation with acetylated or nonacetylated p38 at various concentrations of ATP. The results of this experi-

ment demonstrated that acetylated p38 was able to phosphorylate ATF2 at much lower concentrations of ATP, compared to nonacetylated p38 (Fig. 4C, D, and E). Analysis of these data on a Lineweaver-Burk graph revealed that acetylated p38 has 2-fold-lower K_m for ATP, compared to nonacetylated enzyme. To demonstrate this effect *in vivo*, we overexpressed HeLa cells with p38-HA and/or with PCAF. p38 was immunoprecipitated from these cells and subjected to *in vitro* kinase assay. The data showed that PCAF expression enhanced the activity of p38 to phosphorylate the substrate ATF2 at limiting concentrations of ATP (Fig. 4F and G). The cell lysate from PCAF-alone-overexpressed cells was subjected to IP with HA antibody and used as a negative control (Fig. 4H). These results strongly indicated that acetylation enhances the intrinsic kinase activity of p38 by increasing its affinity for ATP.

p38 is acetylated at K152 and K53 residues. To identify the acetylated lysine residues of p38, *in vivo*-acetylated p38 was

FIG. 3. HDAC3 binds to and deacetylates p38. (A) HeLa cells were cotransfected with indicated plasmids. Cell lysate was subjected to IP with HA antibody, and the resulting complex was analyzed by IB with anti-Flag antibody. Blot was stripped and reprobed with anti-HA antibody. The sizes of the molecular weight markers are indicated on the left. (B) Cells were cotransfected with indicated plasmids, and the cell lysate was subjected to IP with the indicated antibody. The resulting beads were analyzed by IB with the antibody given on the right. (C) HeLa cell lysate was subjected to IP with anti-p38 or anti-HA antibodies, and the resulting beads were analyzed by IB with the use of anti-HDAC3 and anti-p38 antibodies. (D) Cells were cotransfected with indicated plasmids in different combinations. Cell lysate was subjected to IP with HA antibody, and the resulting complex was analyzed by IB with anti-Ac-K antibody. Arrowhead points to acetylated p38 band. Shown at bottom is IB of the WCL depicting input of HDAC1, -3, and -5 in lanes 2, 3, and 4 from the left, respectively. (E) Histidine-tagged p38 on beads was subjected to *in vitro* acetylation using GST-PCAF. Beads were washed and incubated with different HDACs in a deacetylation reaction buffer. Deacetylation of p38 was examined by IB with use of indicated antibodies.

subjected to tryptic digestion and the resulting peptides were analyzed by mass spectrometry. The MS/MS analysis showed that p38 was acetylated at a single lysine residue at position 152 (Fig. 5A). Previous studies have shown that K152 is involved in the substrate binding of p38 (14). Because acetylation can enhance the activity of a protein by increasing its affinity for its substrate, we substituted this lysine (K) with arginine (R) and investigated the impact of this substitution on p38 activity. Substitution of K with R conserves the net positive charge of the amino acid but prevents the neutralization of the charge by acetylation. We found that substitution of K152 with R did not affect the phosphorylation of p38, but this substitution completely abolished the activity of p38 toward the substrate ATF2 (Fig. 5B), suggestive of an essential role for the activity of p38, possibly by affecting the ability of p38 to bind to substrate ATF2.

We then asked whether acetylation had an effect on the affinity of p38 to bind to ATF2. For this experiment, acetylated p38 and nonacetylated p38 were incubated with increasing amounts of ATF2, keeping the excess ATP concentration constant. The idea here was that if the affinity of p38 toward the substrate ATF2 is enhanced, then ATF2 could be phosphorylated, even at its lower concentrations. This, however, did not happen in our assay, and we detected similar amounts of phospho-ATF2 in both acetylated and nonacetylated p38, indicating that protein acetylation did not affect the affinity of p38 for ATF2 (Fig. 5C). To get direct evidence for these findings we tested *in vitro* binding of acetylated and nonacetylated p38 to decreasing amounts of GST-ATF2, and again results showed that there was no difference in the levels of binding of ATF2 to both forms of p38 (Fig. 5D). These results thus demonstrated

FIG. 4. Acetylation enhances kinase activity of p38 by promoting its affinity for ATP. (A) Phospho-p38 (Pho-p38) was acetylated *in vitro* with PCAF. Acetylated Pho-p38 and nonacetylated Pho-p38 were incubated with ATF2 in a kinase buffer containing 200 μ M ATP. At indicated time points, reaction was stopped and samples were analyzed by IB with use of anti-Pho-ATF2 antibody. Blot was stripped and probed with anti-Pho-p38 antibody. Same blot was stained with Coomassie blue to verify equal loading of ATF2. (B) ATP-binding ability of acetylated, nonacetylated, or inhibitor-bound (SB; SB203580) Pho-p38 was determined by incubation with different amounts of [32 P]ATP as indicated. Values are mean $\pm$ SE of 3 experiments. (C) In a kinase buffer, ATF2 (1 μ g) was incubated with acetylated or nonacetylated Pho-p38 at various concentrations of ATP, as indicated. Samples were analyzed by IB with use of anti-Pho-ATF2 antibody. Blot was stripped and probed with anti-Pho-p38 antibody and later stained with Coomassie blue to verify equal loading of ATF2. (D) Experiment similar to that described in the legend to panel C was done with more points analyzed between 50 and 200 μ M ATP. (E) IB showing acetylation of Pho-p38, utilized in assays described in the legend to panels A, C, and D. (F) HeLa cells were overexpressed with HA-p38 with or without PCAF. HA-p38 was subjected to IP and incubated with GST-ATF2 (1 μ g) in kinase buffer with different amounts of ATP as indicated. Samples were analyzed by IB with use of anti-Pho-ATF2 antibody. Blot was stripped and probed with anti-GST, anti-Pho-p38 antibodies, and, later, anti-p38 antibody. (G) Quantification of activity of acetylated and nonacetylated p38. Mean $\pm$ SE; $n = 3$. (H) HeLa cells were cotransfected with indicated vectors. Cell lysate was subjected to IP p38, and the resulting beads were assayed for activity to phosphorylate the substrate ATF2. ATF2 phosphorylation was detected by IB with indicated antibodies.

that the enhanced activity of acetylated p38 was not related to its enhanced affinity toward the substrate.

We then examined the acetylation of p38K152R mutants by IB and found that this mutant was still acetylated, suggesting the possible existence of additional acetylation sites (Fig. 5B, bottom). Because in our previous experiments we found that acetylation enhanced the affinity of p38 for ATP, we anticipated that lysines located in the ATP-binding pocket of p38

(such as K53 or K54) might be acetylated. We, however, could not obtain a peptide corresponding to the ATP-binding pocket of p38 by tryptic digestion, and hence, acetylation of these lysines could not be detected by MS/MS analysis. We therefore studied the role of these lysines (K53 and K54) in the regulation of p38 activity by substitution mutation analysis. We generated three different p38 mutants as GST proteins by substituting K53 and K54 separately or K53 and K54 together with

FIG. 6. Acetylation of p38 at lysine 53 augments its ATP-binding ability. (A) GST proteins (GST, GST-p38, GST-p38K53R, GST-p38K53R,54R, and GST-p38K54R) were subjected to phosphorylation *in vitro* by MKK6 and then tested for their ability to phosphorylate ATF2. The reaction mix was analyzed by IB with use of anti-Pho-p38 and anti-Pho-ATF2 antibodies. For loading control, blot was stained with Coomassie blue. (B) ATP-binding ability of acetylated, nonacetylated, or inhibitor-bound p38 was measured directly by incubating GST proteins with 15 μ Ci [32 P]ATP. Values are mean $\pm$ SE of 3 experiments. (C) HeLa cells were overexpressed with wild-type p38 or p38K152R mutant. p38 was IP, and the resulting beads were assayed for ATP-binding ability by incubating them with 15 μ Ci [32 P]ATP. Beads treated with SB served as a control. Values are mean $\pm$ SE of 3 experiments. (D) His-p38 and His-p38K53R were subjected to acetylation with PCAF. His beads were separated and analyzed by IB. Top portion of the blot was probed with anti-PCAF, and bottom portion with anti-Ac-K and later for anti-p38 antibodies. (E) HeLa cells were overexpressed with indicated plasmids in different combinations. Cell lysate was subjected to IP p38 with HA antibody, and the resulting complex was analyzed by IB with anti-Ac-K and anti-HA antibodies. WCL was also analyzed by IB to determine PCAF expression. (F) Non-phosphorylated and phosphorylated p38 was subjected to acetylation by PCAF *in vitro*. The resulting acetylated p38 was then tested for its ability to phosphorylate the substrate ATF2. The samples were analyzed by IB with use of indicated antibodies.

mutant (the K53R mutant) together with PCAF. The cell lysate was subjected to IP with anti-HA antibody, and the resulting complex was analyzed by IB with anti-Ac-K antibody. Again, we found that wild-type p38 was acetylated *in vivo*, but the K53R mutant showed remarkably reduced acetylation (Fig. 6E). These results thus confirmed that p38 is acetylated at the K53 residue.

Acetylation at lysine residues within a protein is considered to cooperate with or rival phosphorylation. To test whether acetylation of p38 (without phosphorylation) was sufficient for its activation, we incubated phosphorylated and nonphosphorylated p38 with PCAF for acetylation and then examined their ability to phosphorylate the substrate ATF2. We found that p38 which was both acetylated and phosphorylated was capable of phosphorylating ATF2, but its counterpart which was only acetylated, but not phosphorylated, did not phosphorylate ATF2 (Fig. 6F). These results demonstrated

that the acetylation alone is not sufficient to activate p38, but because acetylated p38 had enhanced activity (Fig. 4), it appears that acetylation augments the phosphorylation-mediated activity of the enzyme.

Acetylation enhances the activity of JNK1. To test whether the activity of other stress kinases could be also modulated by lysine acetylation, we compared the ATP-binding sequences of p38 with other MAPKs and found that JNK1 and JNK2 have similar conserved lysine residues within their ATP-binding pockets (Fig. 7A). We therefore examined the effect of protein acetylation on the activity of JNK1. JNK1 was overexpressed with PCAF in HeLa cells, and acetylation of JNK1 was validated by IP of the kinase followed by IB (Fig. 7B). We then tested the ability of acetylated and nonacetylated phospho-JNK1 to phosphorylate the substrate ATF2. The kinase reaction was carried out in the presence of increasing concentrations of ATP, and the phosphorylation of ATF2 was examined

FIG. 7. Acetylation of JNK1 enhances its kinase activity. (A) Table showing conserved lysines (K underlined) in the ATP-binding pocket of p38, JNK1, and JNK2. (B) HeLa cells were transfected with plasmids synthesizing Flag-JNK1 and/or PCAF in different combinations, as indicated. Cell lysate was subjected to IP with Flag antibody, and the resulting complex was analyzed by IB with use of anti-Ac-K antibody. Arrow points to acetylated JNK1 band. The blot was stripped and probed with anti-Flag antibody. (C) Pho-JNK1 was subjected to *in vitro* acetylation with PCAF. In a kinase buffer, acetylated and nonacetylated Pho-JNK1 was incubated with GST-ATF2 with various amounts of ATP as indicated. The reaction was stopped by addition of Laemmli buffer, and samples were analyzed by IB with indicated antibodies.

by IB with appropriate antibodies. As we did for p38, we found enhanced activity of JNK1 after acetylation at much lower concentrations of ATP (Fig. 7C), thus again suggesting that acetylation increased the affinity of JNK1 for ATP. These results together indicated that in addition to phosphorylation, acetylation is another common mechanism regulating the activity of these kinases.

Acetylation sensitizes p38 for apoptosis. Overactivation of p38 is known to induce apoptosis. In order to understand the functional significance of p38 acetylation, we examined its effect on cell survival. HeLa cells were treated with anisomycin (Ani), a potent activator of p38 and JNK, for 4 h in the presence or absence of TSA. The specificity of anisomycin to a kinase was determined by using the kinase-specific inhibitors SB203580 (SB) and SP600125 (SP), which inhibit p38 and JNK, respectively. We found that treatment of cells with Ani alone induced apoptosis in ~55% of cells, whereas the combination of Ani with TSA led to nearly 100% cell death. Treatment with SB or SP inhibitors alone did not prevent the cell death induced by Ani, but cell death induced by a combination of Ani and TSA was reduced significantly by the addition of either kinase inhibitor, and a mixture of both inhibitors reduced cell death further to a level that was comparable to that which occurred with Ani treatment alone (Fig. 8A and B). Similar results were obtained when we knocked down p38 from HeLa cells and treated them with anisomycin in the presence or absence of TSA (Fig. 8C and D). Treatment of these cells with anisomycin resulted in increased p38 acetylation and reduced ATP levels (Fig. 8F and G). This suggested that TSA-mediated acetylation of p38 and JNK had contributed to the increased cell death.

Because HDAC3 was identified as an HDAC which can deacetylate p38, we repeated the same experiment with cells in which HDAC3 was knocked out by the use of plasmid express-

ing shRNA against HDAC3. The results showed that the HDAC3 deficiency augmented the effect of Ani on cell death, which was again blocked by treating cells with SP and SB inhibitors, thus again highlighting the contribution of p38 and JNK acetylation in increased cell death (Fig. 9A, B, C, and D). To further confirm these results, we cotransfected HeLa cells with p38-, PCAF-, and/or HDAC3-expressing plasmids and treated them with Ani for 2 h. K53R mutant p38 (mt-p38) was used as a negative control. The results showed that when treated with Ani, nearly 75% of cells which overexpressed the p38 wild type underwent apoptosis. Cell death which occurred in the presence of mt-p38 was comparable to that seen with the control (30%). When p38 was coexpressed with PCAF, cell death rose to nearly 94%, and it was reduced to almost 52% when p38 was combined with HDAC3 (Fig. 9E, F, and G). These data strongly indicated that p38 activation by acetylation sensitizes cells to apoptosis.

Acetylation enhances p38 activity during hypertrophy of the heart. Cardiac hypertrophy has been shown to be associated with ATP depletion (15). To understand the relevance of our findings to *in vivo* models of cardiac hypertrophy, we infused mice with isoproterenol (ISO) for 7 days. The cardiac hypertrophic response of mice was measured in terms of an increased heart weight/body weight (HW/BW) ratio, myocyte cross-sectional area, and induction of hypertrophic markers, such as ANF, BNP and β MHC, as characterized previously (50). We also measured ATP levels in ISO-treated hearts. The results showed that mice with ISO infusion had an ~50% increase in the HW/BW ratio, which was associated with a 40% reduction in ATP levels (Fig. 10A and B). Because, our *in vitro* experiments showed increased acetylation of p38 under ATP-depleted conditions, we determined p38 acetylation in ISO-infused hearts. p38 was immunoprecipitated from heart lysates and analyzed for protein acetylation by IB. As shown in Fig.

FIG. 8. p38 acetylation promotes stress-induced apoptosis. (A) HeLa cells were treated overnight with 500 nM TSA. Next day, cells were treated with anisomycin (Ani) (500 ng/ml) for 4 h in the presence or absence of TSA. p38 and JNK inhibitors, 5 μ M SB203580 (SB), and 10 μ M SP600125 (SP), respectively, were added either separately or together 1 h prior to treatment of cells with Ani. Extent of apoptosis was measured by estimating the percentage of annexin V-positive cells by FACS analysis. (B) Bar graph shows results (mean $\pm$ SE) of 3 independent experiments. (C) Cells subjected to p38 knockout (KO) were treated with TSA and Ani, as described in the legend to panel A. (D) Quantification of death of cells described in legend to panel C (mean $\pm$ SE; $n = 3$). (E) IB showing knockdown of p38 in HeLa cells. (F) IB showing acetylation of p38 in Ani-treated HeLa cells. Shown in bottom panel is loss of acetylation signal when Ac-K antibody was first incubated with acetyl-BSA, thus validating specificity of the anti-Ac-K antibody used. (G) Quantification of ATP in HeLa cells treated with Ani for the indicated time points. *, statistically significant ($P < 0.05$).

10C, p38 from the lysate of ISO-treated hearts displayed increased acetylation, compared to control hearts; however, no noticeable difference in the phosphorylation of p38 was found. When the same lysate was tested for phosphorylation of the p38 substrate ATF2, increased phosphorylation of ATF2 was noticed, thus indicating that acetylation of p38 in ISO-treated hearts is associated with its increased activity (Fig. 10D).

To further confirm that acetylation increases p38 activity

independent of its phosphorylation status, we studied the activity of p38 in ISO-treated and control hearts. p38 was immunoprecipitated from the heart lysates, and its activity was assayed in an *in vitro* kinase assay at two different concentrations of ATP. The results showed that, compared to controls, p38 immunoprecipitated from a hypertrophied heart had significantly higher activity under limiting concentrations of ATP (50 or 100 μ M) though no difference in p38 phosphorylation was observed (Fig. 10E and F). To further substantiate these find-

FIG. 9. HDAC3 deficiency promotes p38-mediated apoptosis under stress. (A) HDAC3 was knocked down from 293T cells by the use of a plasmid expressing shRNA against HDAC3. Cells were treated with Ani (500 ng/ml) for 4 h. p38 and JNK inhibitors, SB (5 μ M) and SP (10 μ M) were added either separately or together 1 h prior to treatment of cells with Ani. Extent of apoptosis was measured by estimating the percentage of annexin V-positive cells by FACS analysis. (B) Bar graph shows results (mean $\pm$ SE) of 3 independent experiments. (C) IB showing knockdown of HDAC3 in 293T cells. (D) Cell lysate of 293T cells subjected to HDAC3 knockdown was subjected to IP p38. The resulting beads were analyzed by IB with the use of indicated antibodies. (E) HeLa cells were overexpressed with p38 or mutant p38 (mt-p38; p38K53R) alone or together with PCAF or HDAC3. Cells were subsequently treated with Ani (500 ng/ml) for 2 h, and cell death was measured. (F) Bar graph shows results (mean $\pm$ SE) of 3 independent experiments. (G) Expression levels of p38, PCAF, and HDAC3 in HeLa cells used for experiments described in the legend to panel E. *, statistically significant ($P < 0.05$).

ings, we measured the p38 activity in transgenic mouse hearts that overexpressed HDAC3 (53). The results showed that HDAC3-overexpressing hearts had considerably reduced activity of p38, compared to wild-type control hearts, while there was no change in the phosphorylation of p38 (Fig. 10G). p38 was also found to be deacetylated in HDAC3 transgenic hearts (Fig. 10H). These data together indicate that acetylation increases p38 activity in spite of no change in the phosphorylation status of the enzyme.

DISCUSSION

This study was designed to investigate the role of acetylation on the activity of p38. We found that cellular stress is associated with increased protein acetylation and depletion of ATP. p38 was found to be one such kinase which was acetylated and activated under stress conditions. Experiments carried out to demonstrate the mechanism of acetylation-mediated activation of p38 revealed that a conserved lysine residue in the ATP binding pocket of the kinase was acetylated, which yielded increased affinity of the enzyme for ATP binding. The physiological relevance of these findings was corroborated by the

observation that agonist-induced hypertrophied hearts showed reduced cellular ATP levels and enhanced p38 activity, compared to controls, in spite of similar phosphorylation levels. We also identified HDAC3 as the deacetylase which interacts with and deacetylates p38. These data demonstrate that acetylation of a conserved lysine in the ATP binding pocket of kinases plays an important regulatory role in their activity.

Although acetylation of p38 was not previously reported, there were reports suggesting cooperation between p38 and HATs in regulating the activity of the target protein. It has been demonstrated that phosphorylation of p38 regulates the acetylation of NF- κ B p65 by regulating the activity of p300 (47). p38 was also shown to phosphorylate and enhance the acetyl-transferase activity of p300 (5). It is therefore reasonable to believe that there could be a synergistic association between p300/PCAF and p38 or that all these factors could be the part of the same complex, where p38 phosphorylates and activates these acetyl-transferases and they in return activate p38 by acetylation. There are examples in which acetylation and phosphorylation have been shown to cooperate positively to regulate the transcriptional activity of target proteins. Oxidative stress has been shown to induce acetylation and phos-

FIG. 10. p38 is acetylated and activated during cardiac hypertrophy. (A) HW/BW ratio of control (C; vehicle treated) and isoproterenol (ISO)-treated mice. (B) Quantification of ATP levels in the heart lysates of control and ISO-treated mice. Mean $\pm$ SE, $n = 5$. (C) Endogenous p38 was subjected to IP from the heart lysate, and it was analyzed for acetylation and phosphorylation by IB with use of anti-Ac-K, anti-Pho-p38, and anti-p38 antibodies. (D) IB showing ATF2 phosphorylation in hearts of control and ISO-treated mice. Results of two mice in each group are shown. (E) p38 was subjected to IP from heart lysate of control and ISO-treated mice and was assayed for its activity to phosphorylate the substrate ATF2 at 50 and 100 μ M ATP. Phosphorylation of ATF2 was detected by IB with use of anti-Pho-ATF2 antibody. (F) Quantification of p38 activity. Mean $\pm$ SE; $n = 3$. (G) p38 was subjected to IP from heart lysate of control (C) and cardiac-specific HDAC3-expressing transgenic (T) mice and was assayed for its activity to phosphorylate ATF2 at 50 and 100 μ M ATP. Phosphorylation of ATF2 was detected by IB with use of indicated antibodies. (H) IB showing reduced acetylation of p38 in the heart lysate of HDAC3 transgenic mice, compared to controls. *, statistically significant ($P < 0.05$).

phorylation of p53, resulting in transcriptional upregulation of its targets needed to combat oxidative stress or to induce apoptosis (48). p38 has been shown to phosphorylate and activate p53 during radiation-induced stress of MCF-7 cells (7). Similarly, p38-mediated phosphorylation of MEF2 increases its transcriptional activity, whereas acetylation of MEF2 by p300 enhances its DNA binding ability, thereby further enhancing the gene transcription ability of MEF2 (18, 32). This extreme parallelism between protein acetylation and p38-mediated phosphorylation/activation of stress-associated proteins strengthens our observation of the augmented activity of p38 upon acetylation.

The increased activity of HATs and p38 has been implicated in various models of cardiac hypertrophy, in which they either individually or in combination have been shown to regulate the induction of cardiomyocyte hypertrophy. The increased catalytic activity of CBP/p300 was found to be associated with agonist-induced cardiac hypertrophy (16). In agreement with this, overexpression of p300 in cardiomyocytes and cardiac-specific expression of p300 in transgenic mice was shown to result in the development of hypertrophy (17, 57). Also, data obtained from cardiac-specific overexpression of the upstream kinase of p38, MKK3, and MKK6 have been shown to induce hypertrophy and the rapid progression of hypertrophy to heart failure (31). The p38 activation was also reported during pressure overload hypertrophy and with dilated end-stage failing human hearts (8, 21). In contrast, in many other studies, re-

duced or no change in activity of p38 was noticed in different models of hypertrophy (6, 50). These discrepancies could be explained based on our data presented here, because phosphorylation of p38, as it was taken as an indicator of p38 activity in these studies, may not be a true display of its activity. Rather, acetylation of p38 after phosphorylation can enhance the activity of p38 more than two-fold under energy-deprived conditions, which was not measured in these studies in which p38 phosphorylation was shown to be unchanged during cardiac hypertrophy.

As mentioned above acetylation and phosphorylation can cooperate positively or block each other's activity. For example, acetylation of STAT3 after phosphorylation results in its enhanced DNA binding and increased transcriptional activity (54). In contrast, acetylation of lysines in the catalytic domain of CDK9, MKK6, and LKB1 was found to inhibit their kinase activity by blocking their phosphorylation (28, 38, 46). Opposite to this, phosphorylation of forkhead transcription factors and estrogen receptor has been shown to prevent their acetylation (9, 33). In this study, we have identified a new mechanism of synergism between acetylation and phosphorylation to regulate the activity of p38. Our data demonstrate that acetylation of the highly conserved K residue of the ATP binding pocket of p38 enhances the affinity of p38 for ATP binding and thereby increases its enzymatic activity. Because other MAPK kinases also have the lysine residue at identical positions in their ATP-binding pockets, we investigated acetylation of

another MAPK, JNK, and the results showed that, as with p38, acetylation of JNK enhanced its affinity of ATP binding, thereby increasing its kinase activity. These findings have a wide implication in the field of ATP-binding proteins. The sequence GXXXXGKT/S, popularly known as the Walker motif, has a conserved lysine residue, and it is widely believed to be the critical site of ATP binding (44). Previously, mutation of this lysine residue was shown to result in reduction or loss of activity of the protein (24). Acetylation of lysine in the ATP-binding pocket of CDK9 was shown to result in reduced activity of the enzyme. Similarly acetylation of lysine 33 that is involved in the binding with ATP of CDK2 inactivates its kinase activity (34). This is expected, because CDK9 inhibition is known to trigger the upregulation of genes involved in the stress response of the cell, and CDK2 is involved in the progression of the cell cycle (10, 34). On the other hand, acetylation-dependent activation of p53 induces apoptosis. It is therefore very likely that, in general, proteins involved in cell cycle progression get inactivated by acetylation, whereas proteins involved in cell cycle arrest get activated by acetylation. Supporting these observations, our data presented here suggest that acetylation at the conserved lysine in the ATP-binding pocket could be a mechanism by which the activity of an ATP-binding protein is controlled.

Our observation that lysine 152 of p38 is also acetylated warrants further investigation, because this lysine is highly conserved and is necessary for substrate phosphorylation. But in this study we could not attribute any functional significance to K152 acetylation, because mutation of this lysine to either arginine or glutamine (data not shown) abrogated the p38 activity with no effect on its phosphorylation. Acetylation could also change the activity of a protein by altering its stability (25). Traditionally, acetylated proteins have a longer half-life compared to their unacetylated counterparts, because unacetylated lysines are targets of ubiquitination-mediated proteosomal degradation. This possibility was ruled out in our study, because the change in activity of p38 after acetylation was observed with *in vitro* models, which lack ubiquitination machinery. Furthermore, data obtained by studying direct binding of ATP to acetylated and nonacetylated p38 confirmed our observation that acetylation enhanced the affinity of p38 for ATP, leading to activation of the MAPK.

Even though ATP production is an efficient process in the cell, severe oxidative stress is known to cause ATP depletion by hampering the activity of different processes involved in ATP synthesis (1). Also, ATP depletion can occur by hemichannel-mediated loss of ATP and/or by enhanced consumption of ATP to meet the increased metabolic demand of the cell (29). Because cells produce and expend energy at a very high rate, any of the above reasons or a combination of them can cause drastic and rapid depletion of cellular ATP levels during oxidative stress. We observed reduced ATP levels and increased p38 activity in stressed cardiomyocytes as well as in hypertrophied hearts. Partial intracellular ATP depletion of 25 to 70% has been shown to induce apoptosis, whereas more severe ATP depletion, below 15% of the control, resulted in necrotic cell death (2), suggesting that partial ATP depletion can upregulate the stress-activated MAPKs, JNK and p38, often associated with apoptosis. In this study we found a 2-fold reduction in K_m for the acetylated p38. This can have a considerable

impact on the activity of p38, especially when ATP is considered to exist as distinct pools inside the cell. There are data showing that ischemia and anoxia can cause a preferential decrease in cytosolic ATP, compared to mitochondrial ATP, levels. Under anaerobic conditions in which the mitochondrial ATP/ADP ratio decreased 2-fold, the cytosolic ATP/ADP ratio was found to be decreased nearly 20-fold (2). Moreover, in the current study we observed a 90% reduction in ATP levels when HeLa cells were treated with anisomycin, thus suggesting that 2-fold reductions in the K_m of acetylated p38 will have profound effects on the activity of p38 under these ATP-starved conditions.

In summary, we demonstrated a new role of K-acetylation in the regulation of the enzymatic activity of p38. Our findings have wide implications in the field of kinases because almost all known kinases have an invariant lysine residue in the ATP-binding pocket that corresponds to position K53 of p38. Mutation of such a lysine has been shown to result in reduction or loss of activity of the protein (10). We propose here that reversible acetylation at the conserved lysine in the ATP-binding pocket could be a common mechanism by which the activity of an ATP-binding protein is controlled.

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