Histone Deacetylase 3 (HDAC3)-dependent Reversible Lysine Acetylation of Cardiac Myosin Heavy Chain Isoforms Modulates Their Enzymatic and Motor Activity

Sadhana A. Samant 1, Vinodkumar B. Pillai 2, Nagalingam R. Sundaresan 1, Sanjeev G. Shroff 2, and Mahesh P. Gupta 1, 3

From the 4 Department of Surgery, The University of Chicago, Chicago, Illinois 60637 and 6 Department of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Reversible lysine acetylation is a widespread post-translational modification controlling the activity of proteins in different subcellular compartments. We previously demonstrated that a class II histone deacetylase (HDAC), HDAC4, and a histone acetyltransferase, p300/CREB-binding protein-associated factor, associate with cardiac sarcomeres and that a class I and II HDAC inhibitor, trichostatin A, enhances contractile activity of myofilaments. In this study we show that a class I HDAC, HDAC3, is also present at cardiac sarcomeres. By immunohistochemical and electron microscopic analyses, we found that HDAC3 was localized to A-band of sarcomeres and capable of deacetylating myosin heavy chain (MHC) isoforms. The motor domains of both cardiac α- and β-MHC isoforms were found to be reversibly acetylated. Biomechanical studies revealed that lysine acetylation significantly decreased the $K_m$ for the actin-activated ATPase activity of MHC isoforms. By in vitro motility assay, we found that lysine acetylation increased the actin-sliding velocity of α-myosin by 20% and β-myosin by 36% compared with their respective non-acetylated isoforms. Moreover, myosin acetylation was found to be sensitive to cardiac stress. During induction of hypertrophy, myosin isoform acetylation increased progressively with duration of stress stimuli independently of isoform shift, suggesting that lysine acetylation of myosin could be an early response of myofilaments to increase contractile performance of the heart. These studies provide the first evidence for localization of HDAC3 at myofilaments and uncover a novel mechanism modulating the motor activity of cardiac MHC isoforms.

Lysine acetylation is a reversible post-translational modification process in which histone acetyltransferases (HATs) transfer the acetyl moiety from acetyl coenzyme A to the ε-amino group of lysine (Lys) within a protein (1). The opposite reaction is carried out by another group of enzymes called histone deacetylases (HDACs). Although these names suggest that HATs and HDACs are specific for histones, this is not the case. Recently, lysine acetylation of a multitude of non-histone proteins including transcription factors, kinases, microtubules, and mitochondrial proteins has been identified (2). It has also been recognized that convergence between acetylation and other post-translation modifications such as phosphorylation, methylation, ubiquitination, and oxidation can form multisite modification programs to regulate the activity of a target protein (3).

HAT activity is intrinsic to several transcription co-activators including p300, a functional homologue of cAMP-response element-binding protein (CREB)-binding protein (CBP), and p300/CBP-associated factor (PCAF). HATs are generally phosphoproteins, and their activity is regulated by different signalling kinases (4). HDACs belong to a large family of co-repressors, which can be classified into three main classes, I, II and III, based on their homology to three biochemically and structurally different yeast histone deacetylases, Rpd3, Hda1, and Sir2, respectively (5, 6). All members of the HDAC family contain a highly homologous catalytic domain; however, sequences outside the catalytic domain are very divergent, suggesting that these enzymes have different biological functions and a much broader substrate repertoire beyond histones. The class I HDACs (HDAC1, HDAC2, HDAC3, HDAC8, and HDAC11) are ubiquitously expressed and generally localized to the nucleus with the exception of HDAC3 and HDAC8, which are also present in the cytoplasm (7, 8). The class II HDACs (HDAC4, HDCA5, HDAC6, HDAC7, HDAC9, and HDAC10) are generally cytoplasmic and have the ability to shuttle in and out of the nucleus as needed (5). The class III HDACs, also called sirtuins (SIRTs), are NAD-dependent deacetylases. In mammals seven different SIRT isoforms (SIRT1–SIRT7) have
Lysine Acetylation Regulates Myosin Motor Activity

been identified. They are ubiquitously expressed and are implicated in regulation of various biological functions including cell growth, metabolism, and genetic control of aging (9).

Myosin is a motor protein capable of converting biochemical energy (ATP) into mechanical energy by interaction with actin filaments (10). In cardiac myocytes two myosin heavy chain (MHC) isoforms, α and β, are expressed. The α-MHC isoform has higher ATPase activity and accounts for a faster rate of actin-sliding velocity than the β-MHC isoform with low ATPase activity. The relative distribution of the two cardiac MHC isoforms is regulated developmentally and by various pathophysiological stimuli (11). In young adult rodents, the α- to β-MHC isoform ratio is nearly 95:5, which changes with growth and age of the heart (11). During exercise-induced physiologic hypertrophy, there is no significant change in MHC isoform distribution, whereas during pathologic hypertrophy, β-MHC levels are elevated at the expense of the α-MHC isoform. This MHC isoform shift plays a major role in regulating the contractile function of the hearts of rodents (11). However, the functional significance of MHC isoform shift in large mammals is controversial because they express mainly one isoform, β-MHC (12). It is also not known how in a stressed heart, where the MHC isoform shift takes at least a week to manifest, an existing MHC isoform withstands the initial stages of cardiac stress. Many studies conducted with skeletal muscle MHC isoforms have indicated that endurance exercise induces myosin ATPase activity of slow muscle fibers without a change of MHC isoform distributions (13, 14). These studies have also reported that increased ATPase activity of MHC isoforms was unrelated to change in composition and/or phosphorylation of myosin light chains. However, a mechanism other than isoform shift and light chain phosphorylation regulating myosin ATPase activity is not yet understood.

We have previously demonstrated that treatment of skinned fibers with a class I and II HDAC inhibitor, trichostatin A (TSA), increases the contractile activity of myofilaments (15). We have localized PCAF and HDAC4 to different regions of the cardiac sarcomeres and shown that cardiac sarcomeric proteins are acetylated at lysine residues (15). In this study, we report localization of another HDAC, HDAC3, to A-band of cardiac sarcomeres and that the head region of MHCs is reversibly acetylated at lysine residues. We also demonstrate that this post-translational modification decreases the $K_m$ for the actin-activated ATPase of MHC isoforms and increases motility of cardiac myosin motors.

Materials and Methods

Antibodies Used—The following antibodies and conjugates were used in this study: rabbit anti-acetyl-lysine (9441, Cell Signaling Technology/Millipore; 06-933, Upstate/Millipore), goat anti-actin (sc-1616, Santa Cruz Biotechnology), mouse anti-α-actinin (A7811, Sigma), rabbit anti-HDAC3 (ab2379 and ab16047, Abcam), HDAC3 blocking peptide (ab16279, Abcam), anti-histone 2A (H2A) antibody (2578, Cell Signaling Technology), and anti-cardiac MHCs (ab15, Abcam). All other appropriate secondary (conjugated) antibodies used here were as described before (15).

Plasmid Constructs and Reagents—Mouse α- and β-MHC cDNA constructs were a kind gift from J. Robbins, Cincinnati Children’s Hospital, Cincinnati, OH. These constructs were used as a template for PCR to amplify the head domain of mouse α- and β-MHCs corresponding to amino acids 1–800. The amplified PCR fragment of each MHC was cloned in BamHI-SalI sites of pBiEx3 vector (Novagen). Active PCAF and p300 HAT domain proteins were purchased from Upstate/Millipore. All other chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

Animal Studies—Male mice (20–30 g) from CD1 strain were used for all animal experiments. All animal protocols followed in this study were in accordance with the University of Chicago Institutional Animal Care and Use Committee. The iodine-deficient diet purchased from Harlan Teklad (Madison, WI) contained 0.15% polythyiourea (PTU). Mice were fed with PTU diet for 6–8 weeks to replace α-MHC with β-MHC isoform. Miniosmotic pumps (Alzet Model 2002) were implanted in adult littermate mice anesthetized with ketamine (100 mg/kg of body weight) and xylazine (5 mg/kg of body weight). Pumps were filled with either phenylephrine (PE), isoproterenol (ISO), or vehicle (150 mM NaCl and 1 mM acetic acid) and were set to deliver PE at 75 mg/kg/day or ISO at 8.7 mg/kg/day (16). PE pumps were implanted for 14 days in 6-week PTU-fed mice. ISO pumps were applied to mice fed with regular diet, and hearts were harvested at the indicated time points after agonist administration. To produce pressure overload hypertrophy, aortic banding was carried out in adult CD1 mice as described previously (17).

Myosin Extraction from the Mouse Heart—Fresh ventricular tissue from adult mouse heart was homogenized using Polytron PT2100 tissue homogenizer in 2 ml of ice-cold myosin extraction buffer (0.3 M KCl, 0.09 M KH2PO4, 0.06 M K2HPO4, 1 mM MgCl2, 1 mM ATP, 1 mM DTT, 1 mM PMSF, and mammalian protease inhibitor). The homogenate was extracted at 4 °C on a rotator for 1 h and then centrifuged at 140,000 × g for 30 min at 4 °C. Supernatant was diluted 10-fold with ice-cold 1 mM DTT, and myosin was allowed to precipitate on ice for 1 h. Precipitated myosin was collected by spinning at 26,000 × g for 20 min at 4 °C. The pellet was resuspended in 300 μl of resuspension buffer (0.6 M KCl, 25 mM imidazole, pH 7.5, 1 mM EGTA, 4 mM MgCl2, and 1 mM DTT) (18). Protein concentration was estimated from absorbance measurement at 280 nm using an extinction coefficient, $E_{1%}^{280}$ of 5.6. This myosin was used for all assays including ATPase activity and in vitro motility assays. A crude myosin was prepared without addition of PMSF to the buffer according to the method of Uchida et al. (19). This preparation is known to contain full-length MHC (220 kDa) and a cleaved 117-kDa fragment, which constitutes subfragment-1 (S1) of MHC.

For Western blot analysis, myosin was extracted from mouse heart ventricles using a myosin extraction buffer as above containing 50 μM TSA, 50 μM nicotinamide (NAM), and 1 μM acetyl-CoA where mentioned. Homogenates were incubated for 1 h on a rotator and cleared by centrifugation, and supernatants were used for further analyses. Myosin isoform separation was done as described before (17). S-tagged α- and β-MHC (1–800-amino acid) head domain proteins were expressed and
purified from bacteria as described previously (15). Skinned fiber preparations, myofibril protein extraction, Western blot analysis, immunostaining, and immunoelectron microscopy were done as explained elsewhere (15).

In Vitro Acetylation Assay—The acetylation reaction was carried out as follows. Myosin or protein to be acetylated (25–50 μg) was incubated at 30 °C for 30 min–1 h in 1× HAT buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT) containing 50 μM TSA, 50 mM NAM, 1 mM acetyl-CoA, and 50 ng of PCAF/μg of myosin. This protocol was modified according to the requirement of downstream application. When p300 HAT domain was used along with PCAF for acetylation, 25 ng of p300/μg of myosin was used. When myosin was acetylated for ATPase assays, EDTA was excluded from the reaction buffer. For ATPase and motility assays, acetylation of myosin was carried out only for 20 min to avoid losing myosin enzymatic activity. Acetylation of protein was confirmed by Western blotting for each modification of the procedure. Untreated (control) myosin was also incubated at 30 °C in the same buffer for the same incubation period as used for the acetylated counterpart. Deacetylation assays were carried out using active 

Mass Spectrometric Analysis of Acetylated Lysines—To purify in vivo acetylated MHCs, the cytoskeletal protein fraction was extracted from rat neonatal cardiomyocytes cultured for 10 days (15) with 5-h treatment with 5 μM TSA, 50 mM NAM, and 10 μM acetyl-CoA. The acetylated MHC protein was resolved by 10% SDS-PAGE, and the appropriate band was excised from the gel. Tryptic digests of the purified myosin heavy chain isoforms were analyzed by mass spectrometry according to the method described by Chen et al. (21).

Myosin ATPase Assay—The actin-activated MgATPase activity was determined at 30 °C from the rate of orthophosphate (P_i) release in a reaction buffer containing 15 mM KCl, 6 mM MgCl_2, 2 mM EGTA, 20 mM Tris-HCl, pH 7.5, 2 mM Na_2ATP, 50 μg/ml acetylated or control myosin, and variable concentrations of F-actin. Incubation time was 20–25 min. The reaction was stopped by adding an equal volume of 2% SDS (22). Liberated P_i was determined according to the method of Fiske and Subbarow (23). The following equation (Michaelis-Menten-like kinetics) was used to fit experimentally measured actin-activated ATPase activity data at various actin concentrations, [A]: ATPase = (V_{max}[A])/(K_m + [A]) where V_{max} and K_m are the maximal actin-activated ATPase activity and actin concentration corresponding to ATPase activity of V_{max}/2, respectively. Least square estimates of K_m and V_{max} were obtained using the SigmaPlot® software (Systat Software Inc., San Jose, CA).

Sliding Filament Motility Assays—A portion of myosin was purified further for motility assays as follows. 10× molar excess of chicken skeletal muscle F-actin and ATP to a final concentration of 2 mM were added to myosin prepared as above. Actin-bound dead motors were pelleted down by spinning the solution at 90,000 × g for 20 min at 4 °C. Live myosin motor in the supernatant was used for acetylation reaction and motility assays (24). Acetylated myosin and control myosin were concentrated 4-fold using Millipore Microcon columns. Motility assays were performed at 23 °C in flow chambers constructed of a glass slide, two strips of double-sided tape, and a nitrocellulose-coated coverslip. All reagents were prepared in AB buffer (25 mM imidazole, pH 7.5, 25 mM KCl, 1 mM EGTA, 4 mM MgCl_2, and 10 mM DTT). Reagents were added to the flow chamber in 10-μl volumes in the following order: myosin (incubated for 10 min and again another 10 μl for 2 min), 1 mg/ml BSA in AB buffer to block the surface, 200 nM TRITC-labeled phalloidin-stabilized actin, AB buffer, and motility buffer (2 mM ATP, 0.86 mg/ml glucose oxidase, 0.14 mg/ml catalase, 9 mg/ml glucose, and 2% methyl cellulose (400 centipoises) as a crowding agent in AB buffer). All solutions were incubated in the flow chamber for 2 min except the assay buffer washes. A single flow cell was used to collect all the movies for a given condition (acetylated or control). Movies were collected in epifluorescence on a Zeiss Axiovert 200 with an Andor Luca charge-coupled device camera. Movies were 200 frames in length with exposures of either 0.1 or 0.25 s/frame. Three locations were selected in each movie at random for the analysis. Each of these selected areas encompassed ~5% of the visual area of the movie. A filament was counted as moving only if it moved for at least 10 consecutive frames. This method was used starting in frame numbers 1 and 100 of each movie. Filaments that stalled or moved only part of the time were counted as not moving. Distances were calibrated using a micropatterned standard of known dimensions. Filament tracking was performed using ImageJ. Leading or trailing filament ends were manually tracked using the segmented line tool. The line was measured using built-in software. The length was converted from pixels to μm using the calibration from the micropatterned standard. Rates were calculated by dividing the distance moved by the duration of the tracked motion (calculated by the number of frames moved multiplied by the frame rate of the particular movie). Average rates and standard deviations were then calculated. Additional analysis was performed using the Igor Pro software package. Data from the acetylated and control (untreated) motors were compared using histogram analysis and Student’s t test.

Statistical Analysis—Student’s t test and analysis of excess variance were applied to calculate statistical significance between groups.

Results
MHCs Are Reversibly Acetylated by HATs and HDACs—Previously, we have shown that PCAF and HDAC4 are localized to A-band of sarcomeres (15). We therefore explored whether myosin, a constituent A-band protein, could be targeted for reversible lysine acetylation. We found that myosin heavy chains were acetylated when myofibrillar proteins extracted from adult mouse heart were incubated with acetyl-CoA with or without HDAC inhibitors (Fig. 1A). In this experiment, no acetylation of myosin light chains was detected (data not shown). We confirmed the validity of MHC acetylation by blocking the acetyl-lysine antibody with acetylated BSA (Fig. 1B). To examine MHC acetylation in vivo, we utilized primary cultures of cardiomyocytes that were treated with the HDAC inhibitors TSA and/or NAM (a class III HDAC inhibitor). The results showed that myosin prepared from TSA- but not NAM-treated myocytes was substantially acetylated, thus suggesting
Lysine Acetylation Regulates Myosin Motor Activity

the presence of class I and II, but not class III, HDACs on the sarcomeres (Fig. 1C). This MHC acetylation was further enhanced when the myosin preparation was incubated with acetyl-CoA (Ac-CoA) (Fig. 1C). These results are consistent with our earlier observation showing that Ac-CoA treatment alone is sufficient to increase contractile activity of skinned myofibers, thus confirming the presence of a HAT (PCAF) on myofilaments.

HDAC3 Deacetylates MHC—HDAC4, which was previously localized on sarcomeres, has little or no deacetylase activity of its own (25). However, the discovery that TSA (an inhibitor of class I and II HDACs) is capable of increasing contractile activity of skinned myofibers suggests that other members of the HDAC family must also be localized to cardiac sarcomeres. To identify these HDACs, we performed a Western blot analysis of skinned cardiac myofibers with the use of antibodies against different members of the HDAC family. We found that a class I HDAC, HDAC3, was associated with cardiac myofibers (Fig. 2A). To confirm these results, we carried out immunohistochemical analysis of mouse heart sections and cardiomyocytes cultured for 7 days to enhance sarcomeric organization. Confocal imaging of these cells revealed that HDAC3 is localized not only to the nucleus but also to cardiac sarcomeres, which were identified by staining of a Z-disc protein, α-actinin (Fig. 2B). At higher magnification, HDAC3 was found to be highly localized between two Z-discs (Fig. 2B). The validity of HDAC3 staining was confirmed by use of a blocking peptide (Fig. 2C). To substantiate these results, we carried out immunoelectron microscopic analysis of mouse heart sections. The results showed that high density black dots (10 μm) representing HDAC3 were mostly localized to A-band with a few scattered dots present at the Z-disc but not in the I-band region (Fig. 3A). As a negative control, we stained heart sections with HDAC3 antibody blocked with peptide or with anti-H2A antibody, which showed no staining of sarcomeres (Fig. 3, B and C). These results thus demonstrated that HDAC3 is localized to A-band and to a lesser extent on the Z-disc of sarcomeres.

To test whether HDAC3 was capable of deacetylating A-band proteins, we tested reversible acetylation of MHCs. Proteins were acetylated in vitro with PCAF and then tested for deacetylation with different HDACs (HDAC4 and HDAC3) or SIRT1. The results showed that HDAC3 substantially deacetylated MHCs, whereas HDAC4 and SIRT1 did not (Fig. 3D). In this assay, deacetylation of p300 was used as an internal positive control. SIRT1 used in this assay was tested earlier for its deacetylation activity (20). These results demonstrated that PCAF and HDAC3 are the candidate enzymes that regulate acetylation and deacetylation of MHCs.

The Head Region of MHC Is Acetylated—To determine which region of myosin is acetylated (Fig. 4A), we first analyzed a preparation of adult mouse heart myosin that was prepared without...
addition of PMSF to the buffer (19). This preparation is known to contain full-length MHC (220 kDa) and a cleaved 117-kDa fragment, which constitutes S1 of MHC (Fig. 4B, right panel). By Western analysis with anti-acetyl-lysine (Ac-Lys) antibody, we found that both MHC bands (220 and 117 kDa) were acetylated, suggesting that the head region of MHC was a target of lysine acetylation (Fig. 4B, left panel). To further confirm these results, we examined acetylation of in vitro synthesized S1 fragment of MHCs. The results showed that S1 fragments of both α- and β-MHCs (1–800 amino acids) were acetylated by the HAT PCAF (Fig. 4, C and D).

To identify acetylated lysine residues of S1, we carried out mass spectrometric (MS/MS) analysis of MHC acetylated in vitro and in vivo. By MS/MS analysis of cardiac β-MHC (porcine β-myosin, Sigma) acetylated in vitro, we identified Lys-549 and Lys-633 as acetylated residues (Fig. 5). To validate acetylation of these residues in vivo, we prepared acetylated myosin from 10-day-old rat neonatal cardiomyocytes expressing both α- and β- MHC isoforms. MHC was subjected to MS/MS analysis, which again confirmed the presence of both isoforms of myosin in the sample. We found that MHC was also acetylated at Lys-549 in vivo (Fig. 5A). This residue is conserved between both α- and β-MHC isoforms among all species. However, under our culture conditions, we could not detect MHC acetylation at Lys-633 in vivo. These studies confirmed acetylation of the myosin head and suggested that this post-translational modification is likely to be occurring at Lys-549 and Lys-633 residues, which are part of the actin-interacting surface of myosin (Fig. 5, B and C).

Cardiac Stress Induces MHC Acetylation—Having shown that MHCs are acetylated, we next asked whether this post-translational modification of myosin is sensitive to cardiac stress. We subjected rat cardiomyocytes to cyclic mechanical stretch for 4 h. Subsequently, cell lysate was prepared and analyzed by Western blotting with an anti-Ac-Lys antibody. The results showed significant acetylation of cardiac MHCs in response to stretch. We then examined MHC acetylation during pressure-overload hypertrophy of the heart. Adult mice were subjected to a sham operation or thoracic aortic constriction, and MHC acetylation was examined 8 weeks postsurgery. In this experiment neonatal rat heart expressing β-MHC isoform was used as a positive control. As shown in Fig. 6B, sham-operated mouse hearts expressed only α-MHC, whereas the hearts with thoracic aortic constriction expressed both α- and β-MHC isoforms as expected. By Western blotting with anti-Ac-Lys antibody, both α- and β-MHC isoforms were found to be acetylated, and between the two isoforms, β- was acetylated more than the α-MHC isoform (Fig. 6, B and C).

To confirm these findings and to examine stress-dependent acetylation of individual MHC isoforms, we studied young
adult mice for α-MHC and PTU-treated mice for β-MHC. Mice fed with PTU diet for 6–8 weeks had complete replacement of α- with β-MHC isoform (Fig. 7A). Adult mice were infused with a hypertrophy agonist, isoproterenol, for 1–7 days, and a time course study of MHC acetylation was carried out. ISO infusion increased lysine acetylation of α-MHC, progressively reaching a maximum level by the 4th day of treatment (Fig. 7B and C) when no shift in MHC isoforms could be detected. Similarly, β-MHC was found to be acetylated when PTU-treated mice were infused with the hypertrophy agonist PE (Fig. 7D). These experiments confirmed our observation that cardiac stress induces and modulates acetylation of both α- and β-MHC isoforms.

Lysine Acetylation Reduces the $K_{m}$ for the Actin-activated ATPase Activity of MHC Isoforms—We next evaluated whether lysine acetylation could influence the actin-activated ATPase activity of myosin. Fig. 8A shows a comparison of actin-activated ATPase activity between control and PCAF-mediated in vitro acetylated α-myosin of mouse heart. In this experiment, the control myosin and acetylated myosin for each set of experiments were derived from the same purified preparation of myosin. Enzymatic activity was measured for three indepen-
dent myosin preparations. Values for $K_m$ and $V_{\text{max}}$ (mean ± S.E.) of actin-activated ATPase were as follows: $K_m$, $18 ± 4$ (control) and $11 ± 2 \mu M$ (acetylated, Ac); $V_{\text{max}}$, $1.3 ± 0.1$ (control) and $1.1 ± 0.1 \text{s}^{-1}$ (acetylated). The analysis of excess variance showed that although $K_m$ for acetylated myosin was significantly lower ($p = 0.024$) $V_{\text{max}}$ was not affected by myosin acetylation. In this ATPase assay, we also tested the effect of acetylated actin, which did not influence the actin-activated ATPase activity of myosin isoforms (data not shown). These results suggested that lysine acetylation of MHC isoforms apparently increases affinity between myosin and actin molecules. The PCAF-mediated acetylation of MHC isoform was confirmed by Western blotting (Fig. 8B).

Lysine Acetylation Enhances the in Vitro Motility of Actin-Myosin Motor—Next we performed in vitro sliding filament motility assays on acetylated myosin and control myosin to determine whether the change in actin affinity altered the sliding behavior. These assays were performed using non-acetylated myosin and in vitro acetylated myosin prepared from adult mice (α-MHC isoform) and mice treated with PTU (β-MHC isoform). Fig. 9, A and B, show that at 2 mM ATP concentration the acetylated pool of α-MHC isoform showed a 20% increase in sliding velocity above control, whereas for β-MHC isoform there was a 36% increase upon acetylation. Table 1 shows the percent change in motility for each preparation of α- and β-myosins. Student’s $t$ test showed that in both cases the behavior of the acetylated motor population was distinct from the non-acetylated motor population ($p = 7.3 \times 10^{-5}$ for α- and $p = 4.04 \times 10^{-10}$ for β-MHC isoform). It should be noted that the pool of acetylated MHC isoform used in this assay is likely to be a mixture of acetylated and non-acetylated myosins. If so, then that would imply that the effect of acetylation on motor activity of myosin would be higher than what we observed here.

Discussion—In this study, we report two novel findings. First, HDAC3, a class I HDAC, is localized to cardiac sarcomeres, and second, cardiac MHC isoforms are reversibly acetylated at lysine resi-
dues. We also demonstrate that these acetylated lysines are present in the actin-binding interface of the myosin head domain with an apparent effect on the actin binding affinity of myosin motor. Studies carried out to examine functional consequences of MHC acetylation revealed that this modification increases the actin-sliding velocity of myosin motors. Finally, we demonstrated that lysine acetylation of both $\alpha$-MHC and $\beta$-MHC isoforms was sensitive to stress on cardiomyocytes, and it increased proportionately with intensity of stress during hypertrophy of the heart. These studies reveal a novel post-translational mechanism that may modulate contractile function of cardiac actin-myosin motors.

**Role of HDAC3 on Cardiac Sarcomeres**—In our previous studies we have demonstrated that TSA treatment was capable of enhancing contractile activity of skinned myofilaments, suggesting that members of class I and II HDACs are localized to sarcomeres. We reported earlier that a class II HDAC, HDAC4, is localized to the Z-disc and A- and I-bands of sarcomeres (15). Because HDAC4 has little or no intrinsic deacetylase activity (25), it was apparent from TSA experiments that other members of class I and II HDACs are also localized to sarcomeres. In this study by use of different techniques and antibodies from different sources we demonstrated that a class I HDAC, HDAC3, is also present at cardiac sarcomeres. However, although HDAC4 localizes to different regions of sarcomeres (15), we found that HDAC3 was present mainly at the A-band and to a lesser extent at Z-discs of sarcomeres and had the capability to target MHC isoforms. In this study, we also tested sarcomeric localization of other members of class I HDACs including HDAC8, which has been shown before to bind to cytoskeletal proteins (smooth muscle $\alpha$-actin and $\beta$-actin) (8); however, our results were negative, and we found no other class I HDAC binding to sarcomeres besides HDAC3.

**FIGURE 7.** During cardiac stress MHC acetylation precedes isoform shift. A, Coomassie-stained gel showing separation of different isoforms of mouse cardiac MHC. B, control mice were infused with the hypertrophic agonist ISO for 7 days. Animals were sacrificed at different days after ISO infusion, their hearts were isolated, and MHCs were separated. MHC acetylation was examined by Western blotting. The same blot was stained with Coomassie to verify equal protein loading. C, graphical representation of $\alpha$-MHC acetylation after ISO infusion. Two or three mice were used for each time point to quantify (arbitrary units) myosin acetylation (mean $\pm$ S.D. [error bars]). D, PTU-fed mice were infused with PE for 14 days, and $\beta$-MHC acetylation was determined as in B. Results are shown for two mice in each group. IB, immunoblotting; Cont, control.

**FIGURE 8.** Myosin acetylation reduces the $K_m$ of the actin-activated ATPase activity. A, actin-activated ATPase activity of control and in vitro acetylated mouse $\alpha$-MHC isoforms. Activity was measured at 30°C using 50 $\mu$g/ml $\alpha$-myosin and variable concentrations of actin (2–40 $\mu$M). Data sets were corrected for myosin ATPase activity in the absence of actin and background ATPase activity of actin. The data were fitted to Michaelis-Menten-like kinetics to determine $K_m$ and $V_{\text{max}}$. The data of three different preparations of control myosin (CN) and acetylated (Ac) myosin are shown here. The $K_m$ (mean $\pm$ S.E., $n = 3$) for control $\alpha$-myosin is 18 ± 4 $\mu$M, and the $K_m$ for acetylated $\alpha$-myosin is 11 ± 2 $\mu$M. These $K_m$ values are statistically different ($p = 0.024$, analysis of excess variance). However, the $V_{\text{max}}$ for control myosin and acetylated myosin was not significantly changed. B, a representative Western blot showing PCAF-mediated acetylation of $\alpha$-myosin used in our ATPase and motility assays. IB, immunoblotting.
Among class I HDACs, HDAC3 has been shown to exhibit unique properties. HDAC3 can shuttle in and out of the nucleus, whereas other class I HDACs are found primarily in the nucleus (7). HDAC3 also possesses some distinct structural features that are not present in other members of class I HDACs. The catalytic domain of HDAC3 is located much nearer to the C terminus than in other HDACs, suggesting that HDAC3 might possess unique binding partners and activity (7). A recent report shows that HDAC3 localizes at the plasma membrane where it forms a complex with the tyrosine kinase c-Src, adding to its role outside the nucleus (26). In the nucleus, HDAC3 forms a complex with a transcriptional co-repressor, NCoR-SMRT (nuclear receptor co-repressor 1/silencing mediator for retinoic acid and thyroid hormone receptor), that also includes class II HDACs like HDAC4 and HDAC7 (27, 28). Based on this characteristic, it has been suggested that HDAC3 might be functionally related to class II HDACs. Because HDAC4 is a part of the HDAC3 complex in the nucleus and we have found previously that HAD4 is localized at sarcomeres (15), we tested the possibility whether HDAC4 associates with HDAC3 and forms a complex on sarcomeres. However, even after concerted efforts, we failed to co-precipitate HDAC4 with HDAC3 from myofilaments, suggesting that both HDACs might be associating independently on sarcomeres and that they might target different proteins.

Regulation of Actin-Myosin Motor Activity by Lysine Acetylation—We observed that MHC acetylation did not alter the maximal actin-activated ATPase activity ($V_{\text{max}}$). However, acetylation did reduce the actin-activated $K_{\text{m}}$, implying that there is an increase in the apparent affinity between actin and myosin. Because acetylation also increases sliding velocity, we expect that it also decreases the lifetime of the strongly bound actomyosin state. However, because exit from this strongly bound state is not rate-limiting for the ATPase cycle, this change in lifetime would not change the maximal ATPase activity for the acetylated myosin.

In the heart, MHC isoform shift is considered a hallmark of cardiac hypertrophy (11). Although this mechanism plays an important role in regulation of contractile activity of rodent heart, its value in the control of heart function of large mammals remains disputed as they express mainly one isoform, $\beta$-MHC (11). In the human heart, 2–7% $\alpha$-MHC expression has been reported, but its distribution in the myocardium is not yet

---

**FIGURE 9. Acetylation increases motility rate of both $\alpha$- and $\beta$-MHC isoforms in vitro.** In vitro sliding filament motility assays were performed on control and acetylated forms of $\alpha$- and $\beta$-MHC isoforms purified from mouse ventricles. Four independent myosin preparations were used for the assays. In all cases, the acetylation caused an increase in observed sliding filament velocity. A, a representative histogram for control (untreated) and acetylated $\alpha$-MHC isoform (Ac-myosin). These populations were statistically distinct (t test, $p = 7.3 \times 10^{-5}$, $n = 40$ for each population). The velocity of control $\alpha$-myosin is $2.19 \pm 0.1 \mu$m/s, and that of acetylated $\alpha$-myosin is $2.64 \pm 0.1 \mu$m/s, a 20% increase. B, histogram of control $\beta$-myosin and acetylated $\beta$-myosin. These populations were statistically distinct (t test, $p = 4.04 \times 10^{-10}$, $n = 60$ for each population). The velocity of control $\beta$-myosin is $0.87 \pm 0.02 \mu$m/s, and that of acetylated $\beta$-myosin is $1.18 \pm 0.03 \mu$m/s, a 36% increase. The bin width is $0.25 \mu$m/s.

**TABLE 1**

| Preparations | Ac-$\alpha$-myosin | Ac-$\beta$-myosin |
|--------------|---------------------|-------------------|
| 1            | 20                  | 36                |
| 2            | 16                  | 29                |
| 3            | 31                  | 29                |
| 4            | 11                  | 44                |

The first row in the data was used to draw the histograms in Fig. 9.
Lysine Acetylation Regulates Myosin Motor Activity

known (29). In adult rodent hearts, the α- to β-MHC ratio is ~95:5, which is nearly opposite to the human isoform ratio. Recently, it was demonstrated that 5% β-MHC of the adult mouse heart is distributed to specific regions of the myocardium including the base of the heart and the tip of papillary muscles (30). If a similar distribution pattern exists for 2–7% of α-MHC of the human heart then that will indicate that between the two cardiac MHC isoforms the majority of the work of the adult human heart is carried out by modulation of the activity of only one isoform, which is β-MHC. However, how β-MHC of human heart deals with changing work load on the myocardium is not understood. Our results presented here demonstrate that under stress conditions lysine acetylation might adjust the motor activity of the existing MHC isoform, which may be physiologically relevant for the performance of the human heart (and hearts of other large animals) during increased workload where MHC isoform shift does not seem to play a major role (11).

There are many other studies where post-translational modification of MHCs has been suggested to regulate the motor activity of myosin molecule. Roels et al. (13) have reported that endurance training significantly increases myofilament ATPase activity of soleus muscle despite an increase in slow type MHC-I. Similar observations were made by others who reported increased maximal shortening velocity and increased ATPase activity of slow type 1 fibers of the soleus after forced exercise (14). In contrast, during chronic hypoxia, despite the slow-to-fast transition in MHC isoform distribution, no increase in myofilament ATPase activity was noticed, suggesting that some sort of modification in MHC molecules led to the suppression of myofilament ATPase activity (13). Consistent with these findings, Kamitomo et al. (31) have reported that after chronic hypoxia a significant decrease in MHC ATPase activity occurred in ventricles of sheep, although no change in myosin isoform was detected. These reports also indicated that changes in expression of myosin light chain isoforms and their levels of phosphorylation could not explain the dissociation between MHC ATPase activity and MHC isoform distribution (13, 31). Exercise and hypoxia are well established stimuli affecting lysine acetylation of cellular proteins. Our data suggest that some of the effects reported earlier might be linked with lysine acetylation of myosin motors. Although we have not measured the effect of exercise or hypoxia in this study, we have found that cardiac stress induced by hypertrophic agonists increased acetylation of both cardiac MHC isoforms.

In summary, we have demonstrated that cardiac MHCs are acetylated at lysine residues of the head region, leading to a shift in biomechanical activity of myosin motors. Based on our observations, we propose that during mild stress or initial stages of cardiac hypertrophy where MHCs get acetylated, an increase in the affinity of myosin for actin and escalation in actin-sliding velocity may result in a consequent increase in heart function. In rodents, this post-translational modification may be followed by a shift in MHC isoform if there is further progression of cardiac hypertrophy. However, in large mammals where MHC isoform shift is not a major event to regulate myosin ATPase activity, it is likely that MHC acetylation contributes to a spatial or focused increase in the motor activity of myosin. Because the process of protein modification is more economical than an isoform shift, bypassing transcription/translation of the protein, we believe that in higher mammals this mechanism of MHC acetylation may be better adapted to make the system more energy-efficient. Although additional studies, particularly loss of function with lysine mutation, are required to establish a role of acetylation in regulation of MHC motor function, our studies lay the foundation for a novel mechanism modulating the activity of contractile proteins.

Acknowledgments—We thank Dr. J. Robbins for providing mouse α- and β-MHC cDNA plasmids. The myofilament motility assay was done with help from Dr. R. Rock’s laboratory. Identification of acetylated lysine of myosin head by mass spectrometry was done with help from Dr. Y. Zhao’s laboratory. We also thank C. Labno, S. Bond, and Y. Chen for technical assistance in microscopic analyses.

References

1. Kouzarides, T. (2000) Acetylation: a regulatory modification to rival phosphorylation? EMBO J. 19, 1176–1179
2. Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005) Acetylation and deacetylation of non-histone proteins. Gene 363, 15–23
3. Yang, X. J., and Seto, E. (2008) Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol. Cell 31, 449–461
4. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) Signaling chromatin to make muscle. Curr. Opin. Cell Biol. 14, 763–772
5. de Ruiter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S., and van Kuilenburg, A. B. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 370, 737–749
6. Khochbin, S., Verdel, A., Lemercier, C., and Seigneurin-Berry, D. (2001) Functional significance of histone deacetylase diversity. Curr. Opin. Genet. Dev. 11, 162–166
7. Yang, W. M., Tsai, S. C., Wen, Y. D., Fejer, G., and Seto, E. (2002) Functional domains of histone deacetylase-3. J. Biol.Chem. 277, 9447–9454
8. Waltregny, D., Gleenisson, W., Tran, S. L., North, B. J., Verdin, E., Colige, A., and Castronovo, V. (2005) Histone deacetylase HDAC8 associates with smooth muscle α-actin and is essential for smooth muscle cell contractility. FASEB J. 19, 966–968
9. Blander, G., and Guarente, L. (2004) The Sir2 family of protein deacetylases. Annu. Rev. Biochem. 73, 417–435
10. Ruppel, K. M., and Spudich, J. A. (1996) Structure-function analysis of the motor domain of myosin. Annu. Rev. Cell Biol. 12, 543–573
11. Gupta, M. P. (2007) Factors controlling cardiac myosin-isoform shift during hypertrophy and heart failure. J. Mol. Cell. Cardiol. 43, 388–403
12. Clark, W. A., Jr., Chizzonite, R. A., Everett, A. W., Rabinowitz, M., and Zak, R. (1982) Species correlations between cardiac isomyosins. A comparison of electrophoretic and immunological properties. J. Biol. Chem. 257, 5449–5454
13. Roels, B., Reggiani, C., Reboul, C., Lionne, C., Iorga, B., Obert, P., Tanguy, S., Gibault, A., Jougla, A., Travers, F., Millet, G. P., and Candau, R. (2008) Paradoxical effects of endurance training and chronic hypoxia on myofilament ATPase activity. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R1911–R1918
14. Schluter, J. M., and Pitts, R. H. (1994) Shortening velocity and ATPase activity of rat skeletal muscle fibers: effects of endurance exercise training. Am. J. Physiol. Cell Physiol. 266, C1699–C1673
15. Gupta, M. P., Samant, S. A., Smith, S. H., and Shroff, S. G. (2008) HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. J. Biol. Chem. 283, 10135–10146
16. Sundaresan, N. R., Gupta, M., Kim, G., Rajamohan, S. B., Isbatan, A., and Gupta, M. P. (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J. Clin. Investig. 119, 2758–2771
17. Pillai, J. B., Chen, M., Rajamohan, S. B., Samant, S., Pillai, V. B., Gupta,
18. Jacques, A. M., Briceno, N., Messer, A. E., Gallon, C. E., Jalilzadeh, S., Garcia, E., Kikonda-Kanda, G., Goddard, J., Harding, S. E., Watkins, H., Esteban, M. T., Tsang, V. T., McKenna, W. J., and Marston, S. B. (2008) The molecular phenotype of human cardiac myosin associated with hypertrophic obstructive cardiomyopathy. *Cardiovasc. Res.* 79, 481–491

19. Uchida, K., Murakami, U., and Hiratsuka, T. (1977) Purification of cardiac myosin from rat heart proteolytic cleavage and its inhibition. *J. Biochem.* 82, 469–476

20. Rajamohan, S. B., Pillai, V. B., Gupta, M., Sundaresan, N. R., Birukov, K. G., Samant, S., Hottiger, M. O., and Gupta, M. P. (2009) SIRT1 promotes cell survival under stress by deacetylation-dependent deactivation of poly-(ADP-ribose) polymerase 1. *Mol. Cell. Biol.* 29, 4116–4129

21. Chen, Y., Chen, W., Cobb, M. H., and Zhao, Y. (2009) FTMap—a sequence alignment software for unrestricted, accurate, and full-spectrum identification of post-translational modification sites. *Proc. Natl. Acad. Sci. U.S.A.* 106, 761–766

22. Pollard, T. D. (1982) Myosin purification and characterization. *Methods Cell Biol.* 24, 333–371

23. Fiske, C. H., and Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375–400

24. Nagy, S., Ricca, B. L., Norstrom, M. F., Courson, D. S., Brawley, C. M., Smithback, P. A., and Rock, R. S. (2008) A myosin motor that selects bundled actin for motility. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9616–9620

25. Lahm, A., Paolini, C., Pallaoro, M., Nardi, M. C., Jones, P., Neddermann, P., Sambucini, S., Bottomley, M. J., Lo Surdo, P., Carli, A., Koch, U., De Francesco, R., Steinkühler, C., and Gallinari, P. (2007) Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17335–17340

26. Longworth, M. S., and Laimins, L. A. (2006) Histone deacetylase 3 localizes to the plasma membrane and is a substrate of Src. *Oncogene* 25, 4495–4500

27. Fischle, W., Dequiedt, F., Fillion, M., Hendzel, M. J., Voelter, W., and Verdin, E. (2001) Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. *J. Biol. Chem.* 276, 35826–35835

28. Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., and Verdin, E. (2002) Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. *Mol. Cell* 9, 45–57

29. Nakao, K., Minobe, W., Roden, R., Bristow, M. R., and Leinwand, L. A. (1997) Myosin heavy chain gene expression in human heart failure. *J. Clin. Investig.* 100, 2362–2370

30. Krenz, M., Sadayappan, S., Osinska, H. E., Henry, J. A., Beck, S., Warshaw, D. M., and Robbins, J. (2007) Distribution and structure-function relationship of myosin heavy chain isoforms in the adult mouse heart. *J. Biol. Chem.* 282, 24057–24064

31. Kamitomo, M., Onishi, J., Gutierrez, I., Stiffel, V. M., and Gilbert, R. D. (2002) Effects of long-term hypoxia and development on cardiac contractile proteins in fetal and adult sheep. *J. Soc. Gynecol. Investig.* 9, 335–341

32. Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993) Structure of the actin-myosin complex and its implications for muscle contraction. *Science* 261, 58–65

33. Geeves, M. A., and Holmes, K. C. (1999) Structural mechanism of muscle contraction. *Annu. Rev. Biochem.* 68, 687–728