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Protein kinase C-related kinase targets nuclear localization signals in a subset of class IIa histone deacetylases

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ABSTRACT

Class IIa histone deacetylases (HDACs) -4, -5, -7 and -9 undergo signal-dependent nuclear export upon phosphorylation of conserved serine residues that are targets for 14-3-3 binding. Little is known of other mechanisms for regulating the subcellular distribution of class IIa HDACs. Using a biochemical purification strategy, we identified protein kinase C-related kinase-2 (PRK2) as an HDAC5-interacting protein. PRK2 and the related kinase, PRK1, phosphorylate HDAC5 at a threonine residue (Thr-292) positioned within the nuclear localization signal (NLS) of the protein. HDAC7 and HDAC9 contain analogous sites that are phosphorylated by PRK, while HDAC4 harbors a non-phosphorylatable alanine residue at this position. We provide evidence to suggest that the unique phospho-acceptor cooperates with the 14-3-3 target sites to impair HDAC nuclear import.

Structured summary:
MINT-7710106:*HDAC5* (uniprotkb:Q9UQL6) *physically interacts* (MI:0915) with *PRK2* (uniprotkb:Q16513) by *pull down* (MI:0096)

1. Introduction

Chromatin structure can be altered by post-translational modification of ε-amino groups of multiple conserved lysine residues within nucleosomal histone tails [1]. Histone acetyltransferase-mediated transfer of acetyl groups from acetyl coenzyme A to nucleosomal lysines results in charge neutralization of the amino acid, which weakens the interaction of positively charged histone tails with the negatively charged phosphate backbone of DNA, and culminates in chromatin relaxation. Histone deacetylases (HDACs) catalyze removal of acetyl-groups from lysines. HAT and HDAC activity is generally associated with gene activation and gene repression, respectively.

The 18 mammalian HDACs are grouped into four classes on the basis of similarity to yeast transcriptional repressors [2]. Class I HDACs (1, 2, 3 and 8) are related to yeast RPD3, class II HDACs (4, 5, 6, 7, 9 and 10) to yeast HDA1, and class III HDACs (SirT 1–7) to yeast Sir2. Class II HDACs are further divided into two subclasses, IIa (HDACs 4, 5, 7 and 9) and IIb (HDACs 6 and 10). HDAC11 falls into a fourth class [3].

Class IIa HDACs regulate genes involved in a variety of processes, including skeletal muscle differentiation, cardiac hypertrophy as well as neuron and lymphocyte apoptosis. Derepression of class IIa HDAC target genes is accomplished, in part, by signal-dependent phosphorylation of the HDACs at regulatory serine residues that serve as docking sites for the 14-3-3 intracellular chaperone protein [4–6]. These sites are not present in class I, IIb, III or IV HDACs. Phosphorylation-dependent binding of 14-3-3 to class IIa HDACs results in redistribution of HDACs to the cytoplasm. The mechanism for this redistribution involves activation of a CRM1 exportin-dependent nuclear export sequence (NES) in the HDACs and masking of an HDAC nuclear localization signal (NLS) [4,7–9]. Several kinases have been shown to phosphorylate the 14-3-3 target serines in class IIa HDACs [10], including protein kinase D (PKD) [11] and calcium/calmodulin-dependent protein kinase (CaMK) [12].

Alternative mechanisms for regulating class IIa HDAC subcellular localization have been described. Extracellular signal-regulated kinase was shown to associate with HDAC4 and stimulate its nuclear import, although the relevant phospho-acceptor sites were not defined [13]. Additionally, phosphorylation of a serine residue

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in the NLS of HDAC4 was demonstrated to impair its nuclear import [14], and Dyrk-1B was shown to impair class IIa HDAC nuclear import through phosphorylation of a conserved site within the NLS of the HDACs [15]. Oxidation of cysteine residues in HDAC4 has also been linked to phosphorylation-independent nuclear export of this class IIa HDAC [16].

To further define mechanisms for regulating class IIa HDACs, we used human heart explants to identify proteins capable of interacting with a GST-HDAC5 fusion protein matrix. Here, we describe an interaction between protein kinase C-related kinases (PRKs) and HDAC5. A novel phosphorylation site was mapped to the NLS of HDAC5. This site is conserved in HDAC7 and HDAC9, but is absent from HDAC4. The data suggest that phosphorylation of this site impairs nuclear import of HDACs.

2. Methods

2.1. Biochemical purification of HDAC5-associated proteins

Human heart explants from patients with idiopathic dilated cardiomyopathy (~20 g total left ventricular tissue) were homogenized in Tris buffer (50 mM; pH 7.5) containing EDTA (1 mM), NaCl (100 mM), and protease inhibitors. Insoluble debris was pelleted by centrifugation. Soluble proteins were precipitated by sequential exposure to 20%, 40%, and 60% ammonium sulfate on ice for 20 min. After centrifugation, precipitated proteins were resuspended in homogenization buffer and assayed for HDAC5-directed kinase activity, as described below. Protein present in the 60% ammonium sulfate precipitate was fractionated employing POROS anion exchange resin and a BioCAD perfusion chromatography workstation. Proteins were bound to the resin in Tris buffer (pH 7.5) containing 50 mM NaCl and eluted with an increasing linear gradient of NaCl. Fractions 19 and 20, which contained the bulk of the HDAC5-directed activity (data not shown), were pooled and sequentially added to the GST and GST-HDAC5 affinity resins. Resin-bound proteins were eluted with Tris buffer (50 mM; pH 7.4) containing NaCl (0.5 M). Proteins were precipitated using chloroform/methanol solution, resolved by SDS–PAGE, and stained with colloidal Coomassie Brilliant Blue dye. Proteins found in association with GST-HDAC5 but not GST alone were excised from the gel, dried, and rehydrated in sequencing grade trypsin (Promega) for 20 min on ice prior to overnight incubation at 37 °C. As a negative control, the analogous region of the gel from the GST only lane was excised and analyzed in parallel. Tryptic peptides were extracted in 0.1% trifluoroacetic acid concentrated, bound to C_{18} resin, washed in 0.1% TFA, and eluted from the resin with 80% acetonitrile in 0.1% TFA. Eluted peptides were mixed with a matrix of α-cyano-4-hydroxy-cinnamic acid on a Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) plate and peptide mass spectra were acquired using a MALDI-TOF mass spectrometer in the reflector mode. Spectra were calibrated with autolytic products of trypsin and mono-isotopic peptide masses were used to search the National Center for Biotechnology Information database. A truncated form of protein kinase C-related kinase-2 (PRK2) was found in association with GST-HDAC5, but not GST alone.

2.2. Cell culture and DNA transfection

COS cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (10%), L-glutamine (2 mM), and penicillin–streptomycin. For transfection, cells were plated on six-well dishes (5×10^5 cells/well) 1 day prior to transfection with the lipid-based reagent Fugene 6 (Roche). A truncated active form of PRK1 (amino acids 511–942) and the analogous truncation of PRK2 were constructed by PCR with PFU Turbo (Stratagene) and cloned into pcDNA3.1+, based on the work of Ono et al. [17]. Inactive PRK1 contains a glutamic acid substitution in place of the catalytic lysine at position 644.

2.3. In vitro phosphorylation assay with GST-HDAC5 substrates

GST-HDAC5 substrate containing amino acids 218–328 of HDAC5 fused to the carboxy-terminus of GST was generated in the BL21 strain of *E. coli* employing the pGEX-KG bacterial expression vector. Internal deletion mutants of HDAC5 were generated by PCR with PFU Turbo polymerase (Stratagene). GST-HDAC5 protein (~1 μg) was conjugated to glutathione-agarose beads (Amersham) and beads were washed extensively with PBS prior to use in in vitro kinase reactions. For analysis of kinase activity in column fractions, ~1% of each fraction was mixed (2 h, 4 °C, rocking) with GST-HDAC5 beads in 500 μl PBS containing EDTA (1 mM), Triton X-100 (0.1%), PMSF (1 mM) and protease inhibitor cocktail (Roche). Beads were washed twice in with the same buffer and equilibrated with kinase reaction buffer [HEPES (25 mM; pH 7.6) $MgCl_2$ (10 mM), and $CaCl_2$ (0.1 mM). Beads were resuspended in kinase reaction buffer (30 μl) containing 12.5 μM ATP and 5 μCi [γ-^{32}P]-ATP and reactions were allowed to proceed for 30 min at room temperature. Reactions were boiled, and phospho-proteins resolved by SDS–PAGE and visualized by autoradiography. For experiments with recombinant kinases, COS cells were transfected with mammalian expression vectors for constitutively active forms of PRK1, PRK2 or PKC theta or a catalytically inactive form of PRK1 (1 μg each). Forty-eight hours following transfection, protein lysates were prepared in PBS containing Triton-X100 (0.5%), EDTA (1 mM), PMSF (1 mM), and protease inhibitor cocktail (Roche). Lysates were sonicated briefly and clarified by centrifugation. GST-HDAC5 beads were mixed with ~20 μg total COS lysate and kinase reactions performed as described above.

2.4. Kinase assays with peptide substrates

Kinase activity was measured using a biotinylated HDAC5 peptide (Arg-Arg-Lys-Asp-Gly-Thr-Val-Ile-Ser_{291}-Thr-Phe-Lys-Lys-Arg) or the corresponding peptides from HDAC4 (Arg-Arg-Lys-Asp-Gly-Pro-Val-Val-Thr-Ala-Leu-Lys-Lys-Arg), HDAC7 (Arg-Lys-Glu-Ser-Ala-Pro-Pro-Ser-Leu-Arg-Arg-Arg) or HDAC9 (Arg-Arg-Lys-Asp-Gly-Asn-Val-Val-Thr-Ser-Phe-Lys-Lys-Arg) and 96-well streptavidin-coated plates (Promega) and a vacuum system, as previously described [18]. Standard reaction mixtures contained biotinylated peptide (0–40 μM), 50 μM [γ-^{32}P] ATP (~1×10^5 cpm), DTT (1 mM), $MgCl_2$ (10 mM), Hepes buffer (50 mM; pH 7.6) and enzyme solution (0.1 μg) in a final volume of 50 μl. After incubation at 25 °C for 30 min, 10 μl of the reaction mixture was withdrawn and added to streptavidin-coated 96-well plates. Wells were washed successively with 1.2 ml of NaCl (2 M), 1.2 ml of NaCl containing 1% H_3PO_4, 600 μl H_2O and 200 μl 95% ethanol. Plates were allowed to dry in a hood at 25 °C for 1 h and then 25 μl of scintillation fluid (Microscint 20) was added to each well. Incorporation of [γ-^{32}P] ATP into peptide substrates was measured using a Top-Count NXT (Packard). Recombinant PRK1 and PRK2 were purchased from Invitrogen. SAMTM 96-well biotin capture plates were obtained from Promega. ATP and DTT were obtained from Sigma Chemicals. Peptides were purchased from American Peptide Company. [γ-^{32}P] ATP (6000 Ci/mmol) was purchased from Perkin–Elmer.

2.5. GFP-MITR localization studies

Full-length MEF2-interacting transcription repressor (MITR) was fused in-frame to green fluorescent protein (GFP) in the pEGFP-C1 expression plasmid (Clontech). Site-directed mutants were