Histone Deacetylase 6 (HDAC6) Deacetylates Survivin for Its Nuclear Export in Breast Cancer

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Background: Survivin is an oncogenic protein that is acetylated by CBP, which restricts its location to the nuclear compartment and blocks its anti-apoptotic effect.

Results: HDAC6 deacetylates survivin to promote its nuclear exit in estrogen receptor-positive breast cancer cells.

Conclusion: Cross-talk between estrogen, CBP, and HDAC6 regulate the amount of nuclear acetylated survivin.

Significance: Understanding how estrogen regulates survivin nuclear export may influence breast cancer treatment.

Histone deacetylase inhibitors have shown promising early results in the adjuvant treatment of several cancers (1–3). Although there is empiric support for these agents, their mechanism of action in tumor cells is incompletely understood. Histone deacetylase inhibitors block tumor survival pathways at the transcriptional level by inducing expression of tumor suppressor genes such as p53 and p21 as well as by post-translationally modifying non-histone proteins through acetylation of lysine residues (4–6). Global acetylation studies demonstrate that proteins involved in proliferation, cell growth, cell cycle, differentiation, and migration are functionally regulated through acetylation (7). Acetylation/deacetylation alters protein stability, subcellular localization, and subsequent interaction with other proteins. Together, this process can profoundly change protein function, potentially reversing oncogenic potential and increasing tumor susceptibility to treatment.

Our group previously demonstrated that the inhibitor of apoptosis protein survivin is acetylated in a growth factor-dependent manner by the histone acetyltransferase, CREB-binding protein (CBP) in breast cancer cells (8). We showed that CBP-dependent acetylation at Lys-129 maintains survivin protein stability, subcellular localization, and subsequent interaction with other proteins. Together, this process can profoundly change protein function, potentially reversing oncogenic potential and increasing tumor susceptibility to treatment.

In support of this concept, studies have demonstrated that increasing in nuclear survivin promote apoptosis and increase susceptibility of cancer cells to chemical- and radiation-induced cell death (9, 10). Understanding the mechanism of survivin deacetylation could provide new therapeutic targets to inhibit its nuclear export and thereby regulate its anti-apoptotic function.

Histone deacetylase proteins (HDACs) are classified based on their yeast homologues and include class I (HDACs 1, 2, 3, and 8), class IIa (HDACs 4, 5, 7, and 9), class IIb (HDACs 6 and 10), class III HDACs (sirtuins (silent mating type information regulation 2 homolog) 1–7), and class IV (HDAC 11) (1, 2, 11, 12). Except for the class III NAD+-dependent HDACs, the other zinc-dependent HDACs are the current targets of histone deacetylase inhibitor anti-cancer drugs (13). Class I and II

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2 The abbreviations used are: CBP, CREB binding protein; CREB, cAMP-responsive element-binding protein; HDAC, histone deacetylase protein; MEF, mouse embryonic fibroblast; TSA, trichostatin A; LB, leptomycin B.

Received for publication, September 28, 2011, and in revised form, January 31, 2012 Published, JBC Papers in Press, February 9, 2012, DOI 10.1074/jbc.M111.308791

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HDAC6 Restricts Survivin Nuclear Export

HDACs are expressed at high levels in some primary solid tumors, including breast cancer, and have been demonstrated to be directly involved in cancer development in animal models (13).

Through a series of biochemical assays, we identified HDAC6 as a survivin deacetylase that inhibits CBP-dependent survivin acetylation. HDAC6 primarily localized to the nuclear-cytoplasmic junction in quiescent breast tumor cells in culture and in tumor tissue from breast cancer patients. Once activated by CBP in an estrogen-dependent manner, HDAC6 enters the nucleus, promoting survivin deacetylation and nuclear export. Taken together, our findings reveal a novel mechanism of how HDAC6 and survivin interact at the nuclear membrane to control the acetylation state of survivin.

EXPERIMENTAL PROCEDURES

Cells and Culture—HEK293T, HeLa, and MCF-7 cell lines were cultured as described previously (8). mhdac6-null and wild-type mouse embryonic fibroblasts (MEFs) were a kind gift from the laboratory of Dr. Tso-Pang Yao (Duke University Medical Center, Durham, NC). MEF cells were cultured in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. For experiments involving estrogen, MCF-7 cells were cultured for 48 h in phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) containing 5% charcoal-stripped FBS and 1 mM Na3VO4, 1 mM PMSF), scraped, and collected in microcentrifuge tubes (cytoplasmic fraction), and pellets were washed with hypotonic buffer (buffer A; 10 mM Tris, pH 7.5, 10 mM KCl, 0.5 mM EDTA, 1% IGEPA (Nonident P-40)) plus protease inhibitors (1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM DTT, and 1 mM PMSF) and incubated for 15 min at 4°C on a rotating shaker. Lysates were then centrifuged at 14,000 rpm for 20 min at 4°C. Supernatants were collected in separate tubes (cytoplasmic fraction), and pellets were washed with buffer A and recentrifuged as above. Pellets were washed with cold PBS and centrifuged. Pellets were resuspended in high salt hypotonic buffer (buffer B; 20 mM Tris pH 7.9, 25% glycerol, 1.5 mM MgCl2, 400 mM NaCl, and 0.5 mM EGTA) plus protease inhibitors and then incubated for 30 min in 4°C on a rotating shaker. Lysates were then centrifuged, and supernatants were collected in separate tubes (nuclear fraction). Membranes were immunoblotted with antibodies to acetylated survivin (Novus Biologicals), full-length survivin, CBP, HDAC6, Myc, actin (Santa Cruz Biotechnology), FLAG (Cell Signaling), α-tubulin (Sigma), and HDAC1 (Affinity Bioreagents).

Immunofluorescence—Cells were fixed in 3.7% formaldehyde then blocked and permeabilized in 0.1% Triton X-100, 3% BSA in PBS. Primary antibody was rabbit anti-acetylated survivin (Novus Biologicals), mouse anti-survivin (D-8), and rabbit anti-HDAC6 (H-300) (Santa Cruz Biotechnology). Secondary antibody was anti-rabbit IgG conjugated to Dylight 488 and anti-mouse IgG conjugated to Dylight 594 (ThermoFisher Scientific). Slides were mounted with Prolong anti-fade reagent (Invitrogen). Images were captured using a Nikon C1si Confocal microscope.

RESULTS

Survivin Is Deacetylated at Lys-129 by Member of Class I/II HDACs—In previous work, we demonstrated that the histone acetyltransferase protein CBP induced survivin acetylation on multiple lysine residues in the estrogen receptor-positive breast cancer cell line, MCF-7 (8). Through mutational analyses, we also showed that loss of CBP-dependent acetylation at Lys-129 facilitates Crm1-mediated nuclear export of deacetylated survivin, suggesting that survivin acetylation dictates whether it functions as a nuclear or cytosolic protein. To determine the histone deacetylase(s) responsible for deacetylating survivin at this and other residues, we treated MCF-7 cells with either the class I/II inhibitor TSA for 6 h or the class III inhibitor nicotinamide for 24 h, or with both, harvested for total cellular protein, and then performed Western blots using an antibody generated to the specific survivin-acetylated Lys-129 residue or an antibody generated to full-length (total) survivin. In TSA-treated MCF-7 cells, robust levels of acetylated survivin protein were observed (Fig. 1A). By contrast, nicotinamide had no effect on
survivin acetylation, and no additional increases in acetylation were observed after treatment with both TSA and nicotinamide, suggesting that although class I/II HDACs play a role in deacetylating survivin, class III proteins do not. Total survivin was unchanged in either condition when detected with the antibody against the full-length protein, as expected given that acetylated survivin is a small fraction of the total survivin pool. To determine the dose- and time-dependent effects of TSA on survivin acetylation, we treated MCF-7 cells with an antibody to total survivin and to acetylated survivin. Our results demonstrated that 1 μM TSA induced maximal survivin Lys-129 acetylation (Fig. 1B), first detected at 1 h and steadily increasing by 6 h (Fig. 1C), indicating a time- and concentration dependence that should be linked to a functional role for survivin acetylation states. Similar results were also observed in HeLa cells (supplemental Fig. S1).

To demonstrate the effects of HDAC inhibition on the subcellular distribution of survivin, we co-immunostained MCF-7 cells with an antibody to total survivin and to acetylated survivin. In the absence of TSA, a small, basal level of acetylated survivin was detected that localized to the nucleus (Fig. 1D, upper panel). Following TSA treatment, the levels of acetylated survivin significantly increased (~10-fold) in the nuclear compartment (Fig. 1D, lower panel). Total survivin however, was detected in both the cytoplasm and the nucleus of untreated and treated cells. These results were consistent with the Western blot data, suggesting that class I/II HDACs are involved in survivin deacetylation.

**HDAC6 Abrogates CBP-dependent Survivin Acetylation**—CBP is a central histone acetyltransferase that acetylates multiple cancer-associated proteins and thereby alters their activity (11, 14, 15). To demonstrate the CBP-specific effect on survivin acetylation, we transfected HeLa cells with an expression plasmid encoding HA-tagged CBP then immunostained the cells with the survivin-acetylated antibody. CBP highly induced survivin acetylation, and the acetylated survivin protein displayed a nuclear staining pattern after CBP transfection that was similar to that seen following TSA treatment (Fig. 2A). To identify the specific HDAC(s) that deacetylates survivin, we co-transfected HEK293 cells with a Myc-tagged survivin and CBP construct, along with either empty vector, or one of the class I/II HDACs; HDAC1, HDAC2, HDAC3, or HDAC6 and then performed Western blotting on total cell lysates using the survivin-acetylated antibody. The results showed that CBP-dependent survivin acetylation was unaffected by HDAC1, -2, and -3, but was abolished by HDAC6 (Fig. 2B). These results were recapitulated in HeLa cells (Fig. 2C).

**HDAC6 Domain 2 Is Required for Survivin Deacetylation**—HDAC6 is a unique member of the HDAC class IIb family, structurally composed of duplicate catalytic domains and a third ubiquitin-binding domain (11, 16). To identify the domain responsible for the survivin deacetylase activity, we constructed three truncated forms of HDAC6 from the full-length HDAC6 cDNA, corresponding to each domain; histone deacetylase domain 1 (amino acids 1–414), histone deacetylase domain 2 (amino acids 415–903), and ubiquitin binding domain 3 (amino acids 904–1215) (Fig. 2D). We co-transfected HeLa cells with Myc-survivin; HA-CBP; FLAG-HDAC6 domain 1, 2, or 3; full-length HDAC6 or empty vector; with the latter two as positive and negative deacetylase controls, respectively. We performed Western blotting on total cell lysates using the survivin-acetylated antibody. Our results demonstrated that similar to full-length HDAC6, domain 2 alone abolished CBP-mediated survivin acetylation, whereas domains 1 and 3 had little to no effect (Fig. 2E). To further validate that HDAC6 domain 2 is responsible for survivin deacetylation, we utilized an HDAC6 construct (HDAC6-dm) containing a double point mutation that lacks deacetylase activity (12). Our results demonstrated that CBP-dependent survivin acetylation is restored when the HDAC6 deacetylase activity is inactivated (Fig. 2F). Taken together, the data suggest that domain 2 of HDAC6 is necessary and sufficient to inhibit CBP-dependent activity.
survivin acetylation. This is consistent with published reports demonstrating that domain 2 is responsible for the deacetylase activity of other proteins (17, 18).

**HDAC6 Regulates Survivin Deacetylation in Different Subcellular Pools**—As HDAC6 is mainly cytoplasmic but can be stimulated to enter the nucleus via its nuclear localization signal (19), we sought to determine the subcellular compartment(s) within which HDAC6 deacetylates survivin. To examine the endogenous distribution of HDAC6 and acetylated survivin in MCF-7 cells, we performed subcellular fractionation in the presence and absence of TSA. Untreated MCF-7 cells demonstrated a basal level of nuclear acetylated survivin, which significantly increased following TSA treatment (Fig. 3A), consistent with our previous microscopy results (Fig. 1D). HDAC6 primarily localized to the cytoplasm under both conditions. HDAC6 protein levels were not altered by TSA, consistent with our previous microscopy results (Fig. 1D). HDAC6 enters the nucleus to bind survivin and facilitates survivin nuclear export—To further investigate the subcellular region(s) where HDAC6 deacetylates survivin under different conditions, we transfected HeLa cells with and without HDAC6 or CBP and performed immunofluorescence microscopy. In non-transfected cells, endogenous HDAC6 exhibited a perinuclear staining pattern with minimal colocalization with nuclear survivin (Fig. 3C, top row). This pattern was similarly observed after HDAC6 transfection, with a substantial increase in staining noted around the nuclear membrane (Fig. 3C, middle row). The same perinuclear staining pattern was observed in tissue sections obtained from ER-positive breast carcinomas immunostained with anti-HDAC6 (Fig. 3D), supporting the cell culture results. Following CBP transfection in HeLa cells, HDAC6 localized primarily within the nucleus and colocalized with nuclear survivin (Fig. 3C, bottom row). These results show that...
CBP can induce HDAC6 nuclear import, while maintaining acetylated survivin sequestered in the nuclear compartment. Although it has been reported that HDAC6 binds nuclear proteins (20) and that its structure includes an N-terminal nuclear import signal (19), factors regulating its entry into the nucleus have not been identified. The demonstration that CBP can stimulate HDAC6 nuclear entry strongly suggests that HDAC6 itself is modified by histone acetyltransferase proteins.

To establish whether survivin is a direct substrate of HDAC6, HDAC6 was immunoprecipitated from non-transfected HeLa cells or after transfection with HDAC6 or CBP. HDAC6-survivin immunoprecipitants were not well visualized in the non-transfected cells; however, binding between the two proteins was observed in HDAC6-transfected cells and was further increased in CBP-transfected cells (Fig. 3E), consistent with a direct association between HDAC6 and survivin in the nucleus (Fig. 3C).

To examine the potential in vivo requirements for HDAC6 regulating survivin function, we immunostained HDAC6 null and wild-type (WT) MEFs (kind gift from the laboratory of Dr. Tso-Pang Yao, Duke University Medical Center, Durham, NC) with anti-survivin. The localization pattern of survivin differed in these two cell types. Although the WT cells displayed a punctate, cytoplasmic pattern that was excluded from the nucleus (Z-stack, Fig. 3F), the HDAC6 null cells exhibited a primarily nuclear pattern (Fig. 3F), suggesting that HDAC6-mediated survivin deacetylation is required for survivin nuclear export. Collectively, these results support a role for HDAC6 as a guardian or gatekeeper at the nuclear-cytoplasmic border, inhibiting acetylated survivin nuclear export under resting conditions. When stimulated by histone acetyltransferase proteins, HDAC6 is imported into the nucleus and binds survivin to deacetylate the protein as a likely control mechanism to regulate increasing levels of nuclear acetylated survivin and perhaps to increase its cytoplasmic concentration for its anti-apoptotic activity (9).

**HDAC6-mediated Survivin Deacetylation Is Independent of Crm1**—HDAC6, like survivin, is exported from the nucleus by Crm1 (21); therefore, we investigated whether an HDAC6-Crm1 complex is required for survivin deacetylation. Endogenous HDAC6 immunoprecipitants showed strong binding to Crm1 under unstimulated conditions (Fig. 4A). To determine whether Crm1 is required for survivin deacetylation, we treated HeLa cells with the Crm1 inhibitor, leptomycin B (LB), and...
performed Western blot assays for acetylated survivin. HDAC6 inhibited survivin acetylation both in the presence and absence of LB (Fig. 4B), suggesting that Crm1 is not required for survivin deacetylation by HDAC6. These findings were corroborated by immunofluorescence, demonstrating that HDAC6 localizes to the nucleus and colocalizes with survivin under conditions where Crm1 is inactivated by LB (Fig. 4C).

**Estrogen Induces Survivin and HDAC6 Levels and Their Nuclear Localization**—Estrogen was shown recently to increase CBP acetyltransferase activity through coactivator-associated arginine methyltransferase-induced methylation (22). Given our observations that CBP acetylates nuclear survivin, we examined whether estrogen affects survivin expression and localization in MCF-7 cells. Cells were starved for 48 h, treated with 10 nM estradiol or vehicle control for 6 h, and then fixed and co-immunostained with anti-survivin and anti-acetylated survivin. Estrogen treatment mimicked the effects observed previously after CBP transfection (shown in Fig. 2B), promoting an increase in the levels of nuclear acetylated survivin (Fig. 5A). As expected from the observed increase in acetylated survivin, estrogen also enhanced total survivin nuclear localization (Fig. 5B). Interestingly, under conditions of low serum and no estrogen, survivin localized in a punctate, cytoplasmic granular pattern (Fig. 5B), similar to the staining pattern of survivin observed in the HDAC6 WT MEFs, suggesting a potential link between estrogen, survivin, and HDAC6. These effects were also observed when cells were treated with the estrogen receptor antagonist, tamoxifen (supplemental Fig. S2). To determine the effect of estrogen on HDAC6 levels, we performed Western blots on total cell lysates isolated from the estrogen-treated MCF-7 cells. Estrogen treatment was associated with higher levels of HDAC6 as well as acetylated survivin proteins (Fig. 5C), supporting results of clinical studies that show an increase in HDAC6 levels in estrogen receptor-positive breast tumors (23, 24). To examine the effects of estrogen on HDAC6 subcellular localization, we performed immunofluorescence microscopy. In the absence of estrogen, HDAC6 localized to perinuclear regions of the cell. However, after estrogen treatment its localization increased within the nucleus, as demonstrated by z-stack analysis (Fig. 5D). Together, these data show that estrogen treatment leads to an increase in both acetylated survivin and nuclear HDAC6 levels, possibly through increasing CBP activity.

To determine the requirement for CBP in the nuclear induction of HDAC6 in response to estrogen, we knocked down CBP using siRNA in MCF-7 cells. We then isolated cell nuclei and examined the levels of HDAC6 under conditions of low serum and following estrogen treatment. MCF-7 cells transfected with CBP siRNA resulted in an ~90% decrease in nuclear CBP protein (Fig. 5E). Interestingly, estrogen led to an increase in endogenous CBP levels in the nuclei of control siRNA-treated cells, consistent with a stabilization of CBP protein in this com-
partment mediated by estrogen. However, knockdown of CBP in the presence of estrogen resulted in a decrease in HDAC6 protein within cell nuclei (75% of control levels). This finding supports a requirement for CBP in an estrogen-mediated induction of nuclear HDAC6 in MCF-7 cells.

**DISCUSSION**

HDAC proteins have become pervasive cancer treatment targets due to their involvement in multiple signaling pathways that provide a survival advantage for tumor cells (5). The class IIb deacetylase, HDAC6 increases cancer cell motility and promotes metastatic spread in breast cancer models (24, 25). Inhibition of HDAC6 leads to deregulation of acetylation of multiple proteins, resulting in a disruption of microtubule dynamics and aggresome formation, promoting cancer cell death (26). Here, we identified another target for HDAC6, the anti-apoptotic protein survivin. Principally understood as a cytoplasmic protein (27), we demonstrate a novel nuclear role for HDAC6 in deacetylating survivin. Following estrogen-dependent CBP activation, HDAC6 enters the nucleus and interacts with survivin in a mechanism to regulate its acetylation state and promote its nuclear exit.

The multiple functions of survivin are dependent on its highly regulated expression in distinct subcellular pools (28). We showed previously that survivin nuclear export was dependent on its acetylation state (8). Here, we identify that HDAC6-mediated deacetylation promotes its nuclear exit. In wild-type MEFs and in serum-starved breast cancer cells, survivin localizes to cytoplasmic aggregates, whereas in hdac6 null MEFs and estrogen-treated breast cancer cells, survivin localizes diffusely within the nucleus. Although the nature of the survivin cytoplasmic aggregates is yet unclear, HDAC6-dependent aggresome formation exhibits a similar cytoplasmic staining pattern, suggesting they may represent a component of aggresomes or be involved in autophagy (29). Deacetylation of survivin may potentially promote its interaction with α-tubulin, the binding site for which is located within the domain containing amino acids 99–142 (30), which includes the lysine 129 site.

HDAC6 is an estrogen-regulated protein (24) and has been investigated as a potential prognostic factor for ER+ breast cancer. Most ER+ tumors analyzed express HDAC6; however, there is conflicting data on how increased expression may influence patient prognosis. Although Yoshida et al. (31) suggest that high HDAC6 expression correlates with poor prognosis, Zhang et al. (23) report that it correlates with better prognosis. Our results suggest that HDAC6 expression levels may not be as critical as its subcellular localization, providing a potential explanation for previous clinical discrepancies. A distinct perinuclear staining pattern was observed by immunohistochemistry in invasive ductal carcinoma, which correlates with the pattern we observed in untreated breast cancer cells in vitro. By contrast, treatment of breast cancer cells with estrogen stimu-
Figure 6. Model of HDAC6-mediated survivin deacetylation for survivin nuclear export. ER+ breast cancer cells can be stimulated by estrogen that activates the histone acetyltransferase protein CBP. CBP induces HDAC6 expression and promotes its nuclear entry where it deacetylates survivin, independent of Crm1, and stimulates its nuclear export in a mechanism to control nuclear acetylated survivin levels.

Acknowledgment—We thank Ginny Horvasian for help with confocal microscopy.

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