Influenza A virus-induced caspase-3 cleaves the histone deacetylase 6 in infected epithelial cells

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Abstract
Histone deacetylase 6 (HDAC6) is a multi-substrate cytoplasmic enzyme that regulates many important biological processes. Recently, some reports have implicated HDAC6 in viral infection. However, nothing is known about its regulation in virus-infected cells. The data presented here for the first time demonstrate the caspase-3-mediated cleavage of HDAC6 in influenza A virus (IAV)-infected cells. HDAC6 polypeptide contains the caspase-3 cleavage motif DMAD-S at the C-terminus, and is a caspase-3 substrate. The cleavage removes most of the C-terminal ubiquitin-binding zinc finger domain from HDAC6, which could be significant for HDAC6's role in IAV-induced apoptosis in infected cells.

1. Introduction
Histone deacetylases (HDACs), which have been implicated in mediating diverse cellular processes, are divided into three classes. Class I includes HDAC1, 2, 3, and 8, and class II contains HDAC4, 5, 6, 7, 9, and 10. HDAC11 is in a unique class, which shares similarities with classes I and II. Class III comprises the Sir2-like deacetylases SIRT1 to 7. HDAC6 is unique among class II deacetylases because it contains two catalytic domains and a zinc finger motif. HDAC6 is a cytoplasmically localized protein that potentially regulates several cellular functions. In vivo, the enzymatic activity of HDAC6 is exerted on β-tubulin, heat-shock protein 90 (hsp90), and cortactin; whereas, in vitro, HDAC6 also deacetylates histones. In addition to the identification of its enzymatic substrates, several HDAC6-interacting proteins also have been identified (reviewed in [1]).

Besides its role in a wide range of cellular activities, involvement of HDAC6 in viral infections also has been reported lately [1]. HDAC6 regulates the HIV-1 infection by altering the acetylation status of cortical α-tubulin [2]. In human T-cell leukemia virus-infected cells inhibition of HDAC6 activity leads to the enhanced viral gene expression [3]. Because HDAC6 also is involved in actin remodeling underneath the plasma membrane [4], which is the site of influenza A virus (IAV) assembly, we are investigating the role and regulation of HDAC6 during IAV infection. IAV is the prototype of family Orthomyxoviridae and possesses a segmented negative-sense RNA genome. Influenza viruses are among the most common and significant causes of human respiratory infections because of their ability to cause high morbidity and mortality. IAV infects airway epithelium in the upper and lower respiratory tracts. Seasonal IAV strains cause tracheobronchitis and pharyngitis but rarely cause fatal alveolar pneumonia. However, pneumonia is the most common lesion in patients infected with the highly pathogenic avian IAV H5N1 strain [5]. IAV infection results in the induction of apoptosis both in cell culture and in vivo [6–8]. In a recent study, apoptosis was observed among the alveolar epithelial cells of two patients who died of H5N1 infection [9], suggesting that apoptosis may play a role in H5N1 pathogenesis in humans. In this study we demonstrate the caspase-3-mediated cleavage of HDAC6 in IAV-infected cells and discuss its significance.

2. Materials and methods
2.1. Cells, virus, and plasmids
Madin-Darby canine kidney (MDCK) epithelial cells were grown in minimum essential medium (MEM) supplemented with 10%...
fetal bovine serum. Primary normal human bronchial epithelial (NHBE) cells (gift from Greg Conner, University of Miami) were grown in BEMG media (Lonza). Influenza A virus (H1N1) New Caledonia strain was propagated in embryonated chicken eggs. Plasmid pcDNA3 (Invitrogen) expressing human HDAC6 with a C-terminal FLAG tag was provided by Dr. Tso-Pang Yao (Duke University). HDAC6 was cloned at NotI and XbaI sites of pcDNA3. Point mutations in HDAC6 were introduced by QuickChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing.

2.2. Antibodies and reagents

Mouse anti-nucleoprotein (NP) antibody was purchased from Chemicon. Rabbit anti-HDAC6 (amino acid 916–1215) and anti-actin antibodies were purchased from Santa Cruz and Abcam, respectively. Rabbit anti-caspase-3 (cleaved) and anti-FLAG antibodies were obtained from Cell Signaling. Horseradish peroxidase (HRP)-conjugated donkey anti-mouse and anti-rabbit antibodies were obtained from Affinity Bioreagents and Pierce, respectively. Caspase-3 inhibitor Z-DEVD-FMK was purchased from Calbiochem.

2.3. Infection and transfection

For infection, confluent monolayers of cells were washed with phosphate-buffered saline (PBS) supplemented with calcium and magnesium. Virus inoculum, diluted in the PBS and supplemented with 3 μg/ml trypsin was added to the cell monolayers. After 1 h incubation at 37 °C, inoculum was removed and cells were washed once with PBS. Fresh medium was added and cells were incubated at 37 °C. Cells were transfected with the plasmids by Lipofectamine 2000 (Invitrogen) using manufacturer's procedure. Briefly, plasmid DNA and Lipofectamine 2000 were separately diluted in the Opti-MEM I medium (Invitrogen), mixed together and incubated at room temperature for 20 min. The mixture was added to the cells and incubated for 24 h at 37 °C.

2.4. Western blotting

Cells were harvested, washed once with PBS, and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% SDS, 0.5% sodium deoxycholate, and protease-inhibitor cocktail [Roche]). The whole cell lysate was resolved on 10% Bis–Tris or 7% Tris–acetate gels (Invitrogen). Proteins were transferred to nitrocellulose membrane and blocked with 5% nonfat milk in PBS. The membrane was probed with primary antibody followed by secondary antibody at room temperature for 1 h. The antibody dilutions were prepared in blocking solution. The membrane was washed at least three times with PBS-T (0.05% Tween 20) after incubation with each antibody and developed with the Dura chemiluminescent kit (Pierce). The membrane was stripped with Restore buffer (Pierce) before re-probing with another antibody. Films were scanned and images were compiled in Adobe Photoshop CS3.

3. Results

3.1. HDAC6 polypeptide is truncated in infected epithelial cells

We were interested in investigating the regulation of HDAC6 in IAV-infected cells. However, we could not detect the endogenous HDAC6 in MDCK cells with the available HDAC6 antibody. MDCK cells are the best known system for IAV studies. Alternatively, HDAC6 was expressed from a plasmid in MDCK cells, which were then infected with IAV. Most of the HDAC6-expressing cells were infected with IAV (Supplementary Fig. 1). Lysates from such cells were analyzed by Western blotting. A weak or missing HDAC6 band was observed in infected cell lysate as compared to the uninfected cell lysate (Fig. 1a). This indicated the degradation or proteolytic cleavage of HDAC6 in infected cells. However, the polyclonal antibody used to detect the HDAC6 also recognized non-specific proteins besides HDAC6. To confirm this observation, a FLAG antibody was used to detect HDAC6 (plasmid-expressed HDAC6 has a C-terminal FLAG tag). Consistent with the above observation, the FLAG antibody also detected a faint HDAC6 band in infected cell lysate (Fig. 1a), further suggesting its degradation in IAV-infected cells. Because HDAC6 detected here was expressed from a plasmid, we next wanted to find out whether endogenous HDAC6 was undergoing the degradation too. Primary NHBE cells were used to analyze this. As shown in Fig. 1b, endogenous HDAC6 is also truncated in infected cells. To further confirm this finding, a time course study was performed where HDAC6-expressing cells were harvested after 6, 12, and 24 h of infection. Fig. 2a shows that the top band representing HDAC6 disappeared as the infection progressed. Incidentally, disappearance of the HDAC6 band coincided with the cleavage of viral NP (Fig. 2a), which is known to undergo caspase-3-mediated cleavage in infected cells [10]. We confirmed the induction and activation of caspase-3 in IAV-infected MDCK cells by detecting the full-length (procaspase) and cleaved (activated) forms of the protein (Fig. 2b). To assess whether caspase-3 was involved in the degradation of HDAC6, infected cells were treated with the caspase-3 inhibitor Z-DEVD-FMK. Indeed, full length of HDAC6 polypeptide was restored upon caspase-3 inhibition (Fig. 2c). At the same time, cleavage of NP also was inhibited as indicated by its slower mobility in the inhibitor-treated lysate (Fig. 2c, arrows), showing the specificity of inhibitor. These data show that HDAC6 is undergoing caspase-3-dependent cleavage in IAV-infected cells.

3.2. HDAC6 C-terminus contains the caspase-3 cleavage site

Though HDAC6 degradation was inhibited upon caspase-3 inhibition, the cleavage may not be directly mediated by caspase-3
because many proteases are activated during apoptosis in a caspase-dependent manner [11]. To assess whether HDAC6 is a true caspase-3 substrate, we searched the HDAC6 sequence for the presence of putative cleavage motifs. Caspases generally recognize a DXXD motif on their substrates and specifically cleave after second aspartic acid residue [12,13]. After examining the HDAC6 polypeptide we identified two potential caspase motifs, DTYD-S and DMAD-S on the N- and C-termini, respectively (Fig. 3a). To test whether these motifs contain the cleavage site(s), second aspartic acid residues were mutated to glutamic acid. Three HDAC6 variants containing mutations at D172E, D1088E, and D172/1088E positions were created (Fig. 3a), and then expressed in MDCK cells before infecting with IAV. Analysis of infected cell lysates revealed that the DMAD-S motif at C-terminus contained the actual cleavage site (Fig. 3b). Both D1088E and D172/1088E mutants retained their full lengths in infected cells as detected by both HDAC6 and FLAG antibodies, whereas wild-type HDAC6 and D172E mutant were truncated as detected by only HDAC6 antibody (Fig. 3b). Failure of the FLAG antibody to detect wild-type HDAC6 and D172E mutant in infected cell lysates further confirmed that HDAC6 was truncated at C-terminus. Detection of the cleaved NP in respective lysates shows that caspase-3 was active. This data demonstrates that HDAC6 serves as a caspase-3 substrate in IAV-infected cells.

4. Discussion

We have shown here that HDAC6 undergoes caspase-mediated cleavage in IAV-infected cells. The experiments involving the activation and inhibition of caspase-3 in infected cells clearly implicate the caspase-3 in HDAC6 cleavage. Further, like some other caspase-3 substrates [13], DMAD-S cleavage motif identified in HDAC6 contains Met and Ser at -3 and +1 positions, respectively. However, we cannot rule out the involvement of other caspases in HDAC6 cleavage because Z-DEVD-FMK also inhibits caspase-6, -7 and -8. HDAC3 and 4 also have been shown to undergo caspase-dependent cleavage albeit in different systems [14,15]. This report, for the first time, shows the regulation of an HDAC during virus infection. Interestingly, HDAC6 was not cleaved by the recombinant caspase-3 in vitro [15], suggesting that only in vivo-induced caspase-3 is able to cleave it. Cleavage of HDAC3 and 4 promotes their cytoplasmic
localization and apoptosis [14,15]. However, HDAC6 is already localized to the cytoplasm, and the serine-glutamic acid repeat (SE14) domain at C-terminus (residues 888–1024) acts as a cytoplasmic retention signal [1]. Downstream of the SE14 domain HDAC6 contains ubiquitin-binding domain to ubiquitin zinc finger (BUZ) domain (Fig. 4). The caspase-3 cleavage site identified in this study is present between the SE14 and BUZ domains, and cleavage removes most of the BUZ domain from HDAC6 (Fig. 4). As of yet, we do not know the significance of removal of BUZ domain in IAV-infected cells. However, besides being essential for HDAC6 to process the misfolded proteins, the BUZ domain also is required for regulation of hsp90 [4]. Hsp90 has recently been shown to be involved in IAV replication [16]. It is also likely that consequent exposure of SE14 region after BUZ domain removal stabilizes the cytoplasmic retention of HDAC6. Consequently, like HDAC3 and HDAC4, stable cytoplasmic retention of HDAC6 promotes apoptosis in IAV-infected cells, which can be beneficial for IAV replication. It has been reported that inhibition of apoptosis in infected cells significantly impairs the IAV propagation [17].

HDAC6 may play a critical role during IAV replication as all known HDAC6 substrates, α-tubulin, hsp90, and cortactin, are potentially involved in IAV replication [16,18]. IAV replicates in the nucleus and assembles on the plasma membrane of infected cells. IAV particles consist of three major components: the viral envelope, matrix protein (M1), and viral ribonucleoprotein (vRNP) core [19]. During assembly, constituents of viral envelope are directly transported to the plasma membrane via secretory pathway. However, M1, and vRNP constituents NP and RNA polymerase subunits are first imported into the nucleus for vRNP assembly. M1 and vRNP are then exported out of the nucleus and transported to the plasma membrane via an unknown pathway [19]. Hsp90, possibly as a molecular chaperone, regulates the nuclear transport of RNA polymerase subunits [16]. We anticipate that microtubules play a significant role in vRNP trafficking because vRNPs have been colocalized with microtubules [18] and α-tubulin is hyperacetylated in IAV-infected cells (Husain, M., unpublished). Another HDAC6 substrate, cortactin, may be involved in the budding and release of IAV particles by way of its interaction with actin [20]. Previously, actin has been shown to associate with IAV M1 and NP in infected cells [21]. Moreover, HDAC6 also regulates actin remodeling under the plasma membrane [4], the site for IAV assembly and budding. Studies are underway to determine the significance of HDAC6 regulation in IAV-infected cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.feblet.2009.07.005.

References