

Possible Role of Histone H1 in the Regulation of Furin-dependent Proprotein Processing

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Abstract Histone H1 and its C-terminal lysine rich fragments were recently found to be potent inhibitors of furin, a mammalian proprotein convertase. However, its role in the regulation of furin-dependent proprotein processing remains unclear. Here we report that histone H1 efficiently blocks furin-dependent pro-von Willebrand factor (pro-vWF) processing in a dose-dependent manner. Coimmunoprecipitation and immunofluorescence studies confirmed that histone H1 could interact with furin, and the interaction mainly took place on the cell surface. We noted that histone H1 was released from cells undergoing necrosis and apoptosis induced by H₂O₂. Our findings suggested that histone H1 might be involved in extracellular and/or intracellular furin regulation.

Key words furin; histone H1; pro-vWF; proprotein processing

The limited proteolysis of proproteins by proprotein convertases (PCs) is an important cellular event implicated in both homeostasis and in diseases [1]. Furin (EC 3.4.21.75, *FURIN*) was the first identified and the most extensively studied mammalian PC, responsible for the activation of substrates ranging from blood clotting factors, serum proteins, growth factors and hormone receptors to matrix metalloproteinases, viral coat proteins and bacterial exotoxins [2]. Thus, furin is an attractive therapeutic target for intervention in many diseases. Several inhibitors against furin have been designed. Two of the most potent are the peptidyl inhibitor decanoyl-Arg-Val-Lys-Arg-CH₂Cl and the bioengineered serpin protein α 1-Antitrypsin (AT) Portland (known as PDX) [3]. In addition, there are other reported inhibitors: polyarginines [4], *Drosophila* Serpin 4 [5], eglin c mutants [6] and the barley serine proteinase inhibitor 2-derived cyclic peptides [7]. Some inhibitors have been used to prevent the activation of bacterial toxin, the processing of envelope glycoprotein in viral replication and the metastasis of cancer [8–10].

Our previous studies have identified that histone H1 is a potent furin inhibitor with a *K_i* value of 460 nM. Its inhibitory activity relies on its C-terminal lysine-rich domain, composed of approximately 100 residues, and the inhibitory reactive site of this domain has also been pinpointed [11]. Compared with other furin inhibitors, histone H1 is more attractive, both from a basic research view and in terms of its application potential, as it is an endogenous and nontoxic protein that might be more suited to use as a therapeutic agent. It has been shown that furin can process an extensive range of precursor proteins in the *trans* Golgi network (TGN)/biosynthetic and endocytic pathway, at the cell surface and possibly within the extracellular matrix following shedding. It remains to be determined whether proteolytic cleavage of extracellular matrix substrates for furin is carried out by the membrane anchored or the shed form of furin. Many studies have shown that the shedding of furin, which occurs rapidly and intracellularly, might depend on the integrity of the conformation of the enzyme's cysteine-rich region [12]. Although histone H1 normally binds to the linker DNA and is located in the nucleus, it was reported that histone proteins could be located on the surface of various cells [13,

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14]. Moreover, added histones including H1 were found to be able to penetrate through the plasma membrane into HeLa and Colo-205 cells, and the cellular uptake was mostly due to direct translocation through the cell plasma membrane, not to the endocytosis [15]. Therefore, we are interested in whether the extracellular histone H1 could regulate furin activity at the cell surface or in the extracellular matrix.

In this study, the efficacy of histone H1 in cellular inhibition of proprotein processing was demonstrated. Our results showed that the processing of pro-von Willebrand factor (pro-vWF) by furin was clearly blocked by histone H1 in a dose-dependent manner. In addition, histone H1 could be co-immunoprecipitated with furin and their sub-cellular interaction might take place mainly on the cell surface, as shown by immunofluorescence. During cell necrosis and apoptosis induced by H_2O_2 , histone H1 was markedly released to outside cells, facilitating possible interaction with furin. This is the first report to suggest that histone H1 might be involved in the regulation of furin-dependent proprotein processing.

Materials and Methods

Materials

The monoclonal antibody (mAb) against histone H1 (catalog number: sc-8030) and the secondary antibodies (catalog numbers: sc-2004, sc-2005) were purchased from Santa Cruz Biotechnology. The polyclonal antibodies (pAb) against furin (catalog number: ALX-210-134) and vWF (catalog number: Nr.A 0082) were from Alexis and DakoCytomation, respectively. The mAb against VSV tag was from Sigma-Aldrich (catalog number: V 5507). Protein G Sepharose and [35 S]Met/Cys mixtures were from Amersham Pharmacia. The plasmid pCMV-furin was a kind gift from Dr. G. THOMAS (Oregon Health Sciences University, Portland, USA). The full-length cDNA of human vWF (plasmid pSVHVWF1.1) was a kind gift of Dr. Evan J. SADLER (Washington University, St. Louis, USA).

DNA construction and transfection

The cDNA of furin was sub-cloned into the pCDNA3/VSV expression vector (Invitrogen) between the *Eco*RI and *Xba*I sites using the 5' primer 5'-CGGAATTCATGGAGCTGAGGCCCTGGTTGCTA and the 3' primer 5'-GCTCTAGAGAGGGCGCTCTGGTCTTTGATAAA. Cells cultured in 6-cm dishes were transfected using

Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Inhibition of furin-dependent pro-vWF processing in BHK-21 cells

BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 . When cells grew to 90% confluence, 4 μ g of plasmid pSVHVWF1.1 for the expression of human vWF was transfected into BHK-21 cells. To monitor the effect of histone H1 on furin-dependent pro-vWF processing, different concentrations of histone H1 were added to the medium on 6-cm plates 4 h before pulse labeling by the addition of [35 S]Met/Cys mixtures (150 μ Ci) for 1 h. The medium was collected and immunoprecipitated with 1 μ l of anti-vWF polyclonal antibody overnight. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dried, and visualized by autoradiography.

Co-immunoprecipitation and immunoblot

HeLa cells with 80% confluence were treated with ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% deoxycholic acid sodium) containing a cocktail of protease inhibitors (Roche). The pre-cleared lysates were incubated with 2 μ g anti-furin antibody and 20 μ l protein G Sepharose at 4 °C overnight with end-over-end rotation. The precipitated immune complexes were separated on 10% SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes, incubated with either rabbit anti-furin or mouse anti-histone H1 (diluted 1:1000) and the corresponding horseradish peroxidase-conjugated secondary antibodies, visualized with enhanced chemiluminescence (Pierce).

Immunofluorescence

Cells grown on slides were transiently transfected with pCMV-furin, pCDNA3-furin-VSV or pSVHVWF1.1. Forty-eight hours after transfection, the cells were treated with histone H1 at a final concentration of 10 μ M for 30 min, then washed with PBS and fixed in ice-cold methanol for 5 min. After incubation with corresponding primary antibodies, the slides were rinsed in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) and incubated with Tetra methyl Rhodamine Iso Thio Cyanate (TRITC)-conjugated and fluorescein-isothiocyanate (FITC)-conjugated secondary antibodies. Images were captured using a Charge Coupled Device camera (model DC350F; Leica) on a microscope (model DM5000B; Leica).

Necrosis and apoptosis assay

HeLa cells were cultured in DMEM with 10% FBS to 80% confluence, then cultured in FBS-free DMEM. To induce necrosis and apoptosis, H_2O_2 with indicated concentrations were added to cells for 16 h as previously described [16]. In accordance with the manufacturer's instructions, cells were stained with annexin-V FITC for apoptosis detection and propidium iodide (PI) for both apoptosis and necrosis detection using the Annexin V-FITC Apoptosis Detection Kit (Alexis), then analyzed by flow cytometer (Becton-Dickinson FACSCalibur) using CellQuest software (version 3.3). The percentage of dead cells was calculated as a ratio of cells undergoing apoptosis (annexin-V positive and PI negative cells) and cells undergoing necrosis (both annexin-V and PI positive cells) to the total number of cells. At least 50,000 cells were examined for each sample.

Results

Inhibition of cellular processing of pro-vWF by histone H1

It was reported that pro-vWF is processed by furin during the transit of the secretory pathway [17]. Furin and pro-vWF were co-expressed in BHK cells and pulse-labeled as described in "Materials and Methods". When only pro-vWF was transfected, both pro-vWF and its mature-vWF could be found [Fig. 1(A), lane 2], the latter apparently resulting from the processing by the endogenous furin. In contrast, the co-transfection with furin resulted in the disappearance of pro-vWF, indicating a complete conversion of pro-vWF to the mature form [Fig. 1(A), lane 3].

To examine whether histone H1 could regulate the furin intracellular processing of protein precursors, 48 h after transfection with pro-vWF, histone H1 with different concentrations (1 μ M, 10 μ M and 50 μ M) were added to the culture medium for an additional 4 h. As shown in Fig. 1(B), addition of histone H1 led to a decrease in mature vWF. At a concentration of 10 μ M of histone H1, a minor portion of mature vWF could still be detected, whereas the maturation of vWF was completely suppressed by 50 μ M of histone H1. The changes in the ratio of pro/mature vWF indicated an efficient block of pro-vWF maturation in a histone H1 dose-dependent manner.

Interaction of furin with histone H1

In order to examine the possible interaction between H1

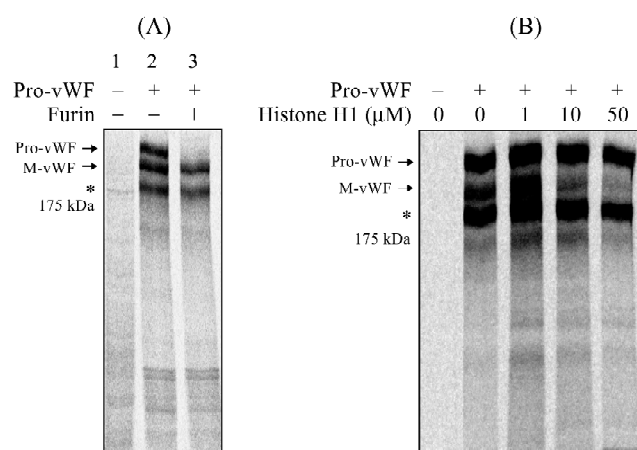


Fig. 1 Inhibition of the furin-dependent pro-von Willebrand factor (vWF) processing by histone H1 in BHK-21 cells

(A) The post-translational processing of pro-vWF by furin. After pulse labeling and a 4 h chase, the medium was immunoprecipitated with anti-vWF polyclonal antibody. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6%) and autoradiographed. 1, mock transfection; 2, transfection with pSVHVWF1.1 for gene expression of pro-vWF; 3, co-transfection with pSVHVWF1.1 and pCMV-furin. The arrowheads indicate the pro-vWF and mature vWF (m-vWF). A major undefined protein, indicated by an asterisk, could also be precipitated with the antibody against vWF. (B) The furin-dependent pro-vWF processing was inhibited by histone H1 in a dose-dependent manner. After transfection with pSVHVWF for 36 h, histone H1 was added to the medium with the indicated final concentrations (1 μ M, 10 μ M and 50 μ M). After 4 h, labeling was carried out for 1 h and the chase for another 4 h. The medium was immunoprecipitated with anti-vWF polyclonal antibody. Immunoprecipitates were analyzed by SDS-PAGE (5%) and autoradiographed. The arrowhead shows the pro-vWF and mature vWF (m-vWF), and the asterisk indicates an undefined protein.

and furin, we investigated whether histone H1 could be co-immunoprecipitated with furin *in vivo*. The whole HeLa cell lysates were immunoprecipitated with an equal amount of normal rabbit immunoglobulin (Ig) G as a control, or polyclonal anti-furin, followed by immunoblot analysis using a monoclonal anti-histone H1, and reprobed with furin antibody. As shown in Fig. 2(A), histone H1 could be co-immunoprecipitated with furin in the HeLa cells, whereas histone H1 could not be detected in the control with the rabbit IgG immunoprecipitation. Conversely, furin could be co-immunoprecipitated with histone H1, but not with the control mouse IgG in HeLa cells [Fig. 2(B)]. The same results of co-immunoprecipitation of furin with histone H1 were also found in BHK21 cells (data not shown). These results imply a possible interaction between histone H1 and furin *in vivo*.

Subcellular co-localization of furin with histone H1

To investigate the subcellular location of the interaction

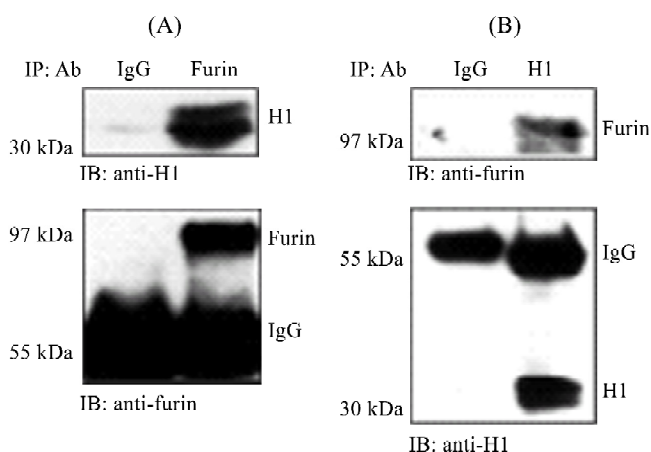


Fig. 2 Co-immunoprecipitation of furin with histone H1

(A) The cell lysates of HeLa cells were immunoprecipitated with a control rabbit immunoglobulin (Ig) G or a polyclonal antibody against furin. After 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, the immunoprecipitates were transferred to the polyvinylidene difluoride membrane and immunoblotted with a monoclonal antibody (mAb) of histone H1 (top) and a polyclonal antibody (pAb) of furin (bottom). (B) The cell lysates of HeLa cells were immunoprecipitated with a control mouse IgG or a monoclonal antibody against histone H1. Using the same approach, the immunoprecipitates were immunoblotted with anti-furin (top) and anti-histone H1 (bottom). IB, immunoblotted; IP, immunoprecipitated.

between histone H1 and furin, immunocytochemistry fluorescent staining was carried out. HeLa cells co-transfected with pcDNA3-furin-VSV and pSVHVWF1.1 were stained with anti-VSV mAb and anti-vWF pAb [Fig. 3(A)]. The staining pattern of furin was the same as that described previously, in that furin was mainly confined to the Golgi complex, and could scarcely be detected on the cell surface [18]. vWF was mainly concentrated at the Golgi complex, but could also be detected on the cell surface [Fig. 3(A)].

In order to examine whether histone H1, when added to the culture medium, translocated across the cell membrane, as many other positively charged DNA-binding proteins would [19,20], HeLa cells were treated with histone H1 (10 μ M), fixed and stained with anti-H1 mAb as shown in Fig. 3(B). When treated with histone H1 for 30 min, both the cell membrane and some of the intracellular cytoplasm were apparently stained. These results indicated that histone H1, added to the medium, could rapidly adhere to and translocate across the cell membrane.

To further confirm the subcellular interaction of furin with histone H1, their immunofluorescence staining patterns [Fig. 3(C)] were compared with those of vWF and histone H1 [Fig. 3(D)]. HeLa cells transfected with furin or pro-vWF were treated with histone H1 (10 μ M)

for 30 min, stained with anti-furin pAb and anti-H1 mAb [Fig. 3(C)] or anti-vWF pAb and anti-H1 mAb [Fig. 3(D)]. The overlapping yellow spots, shown in the figures, were mainly found on the cell surface and some appeared inside the cells, indicating the subcellular co-localization of furin, vWF and histone H1.

Release of histone H1 from cells undergoing necrosis and apoptosis

To study whether histone H1 could be released from cells under physiological or pathological conditions, we examined the release of histone H1 from cells undergoing cell death. H_2O_2 was used because it can induce cell death by either apoptosis or necrosis [16]. As expected, H_2O_2 dose-dependently increased cell death when analyzed by fluorescence-activated cell sorting after staining with annexin-V FITC and PI [Fig. 4(A)]. The corresponding serum-free culture media were immunoprecipitated with an equal amount of anti-histone H1 and analyzed by immunoblot. As shown in Fig. 4(B,C), a small amount of histone H1 could be detected in the serum-free culture media in the absence of H_2O_2 , which indicated that in normal cells histone H1 could be released outside the cells. When cell death increased, histone H1 noticeably released from cells [Fig. 4(B)]. The quantity of released histone H1 was calculated from three independent experiments. It was noted that the relative amount of histone H1 released in the presence of 2 mM H_2O_2 was approximately 4-fold higher than that in the absence of H_2O_2 [Fig. 4(C)]. This result indicated that, in cells undergoing apoptosis and necrosis, more histone H1 was released outside the cells, leading to possible interaction with furin.

Discussion

Limited proteolytic processing plays a central role in homeostasis and in diseases, and these processes must be stringently controlled. Some endogenous inhibitors, such as 7B2 against PC2 [21] and proSAAS against PC1 have been reported [22]. Furin has become a therapeutic target because of its critical role in many cellular events associated with infections such as HIV [23] and SARS [24], activation of bacterial exotoxins [25] and metastasis of cancer [8], as well as the development of atherosclerosis [26] and Alzheimer's disease [27]. PI8 [28] and histone H1 C-terminal fragments [11] are known as possible mammalian endogenous inhibitors of furin, but their physiological role as furin inhibitors has not been fully investigated. PI8 is a broad-spectrum serine protease inhibitor, and the re-

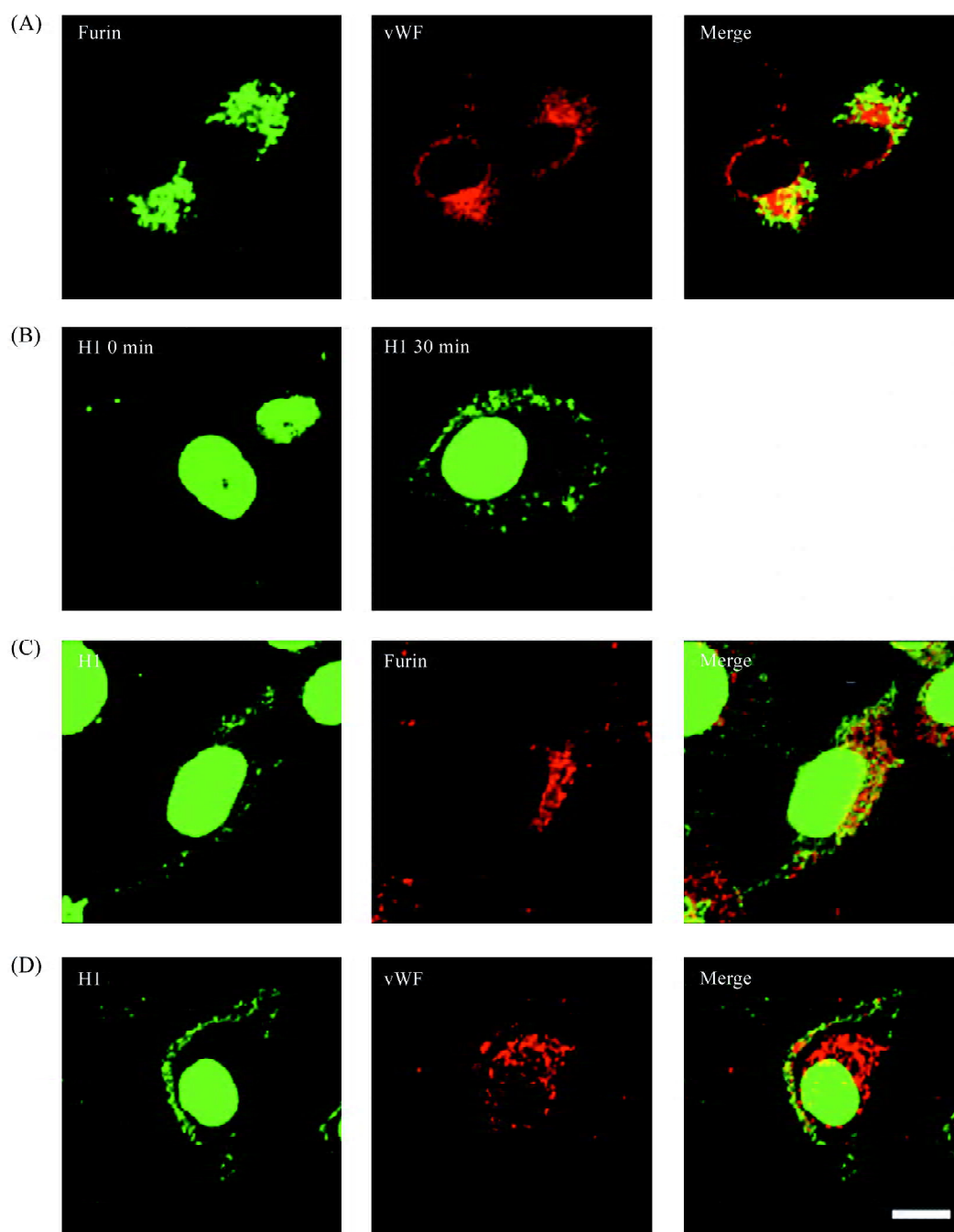


Fig. 3 Subcellular co-localization of furin with histone H1

(A) Co-localization of furin with its substrate von Willebrand factor (vWF). HeLa cells were co-transfected in a 35-mm dish with 1 μ g pcDNA3-furin-VSV and 1 μ g pSVHVWF1.1 for 48 h, then fixed and incubated with anti-VSV monoclonal antibodies (mAb) and anti-vWF polyclonal antibodies (pAb) for 3 h. After washing three times, the slides were incubated for 1 h with Tetra methyl Rhodamine Iso Thio Cyanate (TRITC)-conjugated goat anti-rabbit IgG and fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) G. (B) Histone H1 added to the medium rapidly adhered to and translocated across the cell membrane. HeLa cells were treated with histone H1 to a final concentration of 10 μ M for 30 min, fixed at the indicated time, and stained with anti-histone H1 mAb for 3 h. After washing three times, the slides were incubated for 1 h with FITC-conjugated goat anti-mouse IgG. (C) Co-localization of furin with histone H1 in HeLa cells treated with histone H1. After transfection with 2 μ g pCMV-furin for 48 h, HeLa cells were treated with histone H1 to a final concentration of 10 μ M for 30 min, the fixed cells were stained with anti-furin pAb and anti-histone H1 mAb for 3 h. After washing three times, the slides were incubated for 1 h with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG. (D) Co-localization of vWF with histone H1 in HeLa cells treated with histone H1. After transfection with 2 μ g pSVHVWF1.1 for 48 h, HeLa cells were treated with histone H1 to a final concentration of 10 μ M for 30 min, and the fixed cells were stained with anti-vWF pAb and anti-histone H1 mAb for 3 h. After washing three times, the slides were incubated for 1 h with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG. Scale bar, 15 μ m.

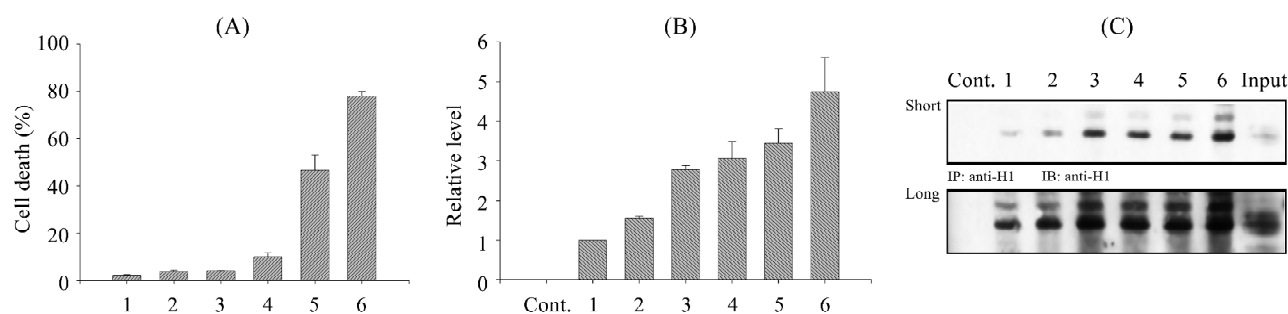


Fig. 4 Release of histone H1 from cells undergoing necrosis and apoptosis

(A) Effects of varying concentrations of H_2O_2 on the death of HeLa cells. HeLa cells were cultured with H_2O_2 of different concentrations for 16 h in serum-free DMEM. The degree of cell death was calculated as a ratio of necrosis cells (annexin- V^+/PI^+) and apoptosis cells (annexin- V^+/PI^-) to the total number of cells/culture. Data were expressed as mean $\pm$ SE from three independent experiments. (B) After 16 h culture with different concentrations of H_2O_2 on 60-mm dishes, the 1.5 ml culture media (serum-free DMEM) of HeLa cells were immunoprecipitated with monoclonal antibody against histone H1, and immunoblotted with anti-H1. To clearly visualize the dose-dependent effect, we have shown the results with different time exposures (exp.). IB, immunoblotted; IP, immunoprecipitated. (C) The quantity of immunoprecipitated histone H1 was calculated using Quantity One software, version 4.4.1 (Bio-Rad). Data were expressed as mean $\pm$ SE from three independent experiments. The control sample was taken for culture without HeLa cells, whereas the input showed the sample from the whole cell lysate. 1, 0 μM H_2O_2 ; 2, 100 μM H_2O_2 ; 3, 200 μM H_2O_2 ; 4, 500 μM H_2O_2 ; 5, 1000 μM H_2O_2 ; 6, 2000 μM H_2O_2 .

combinant PI8 is able to inhibit porcine trypsin, human thrombin, human coagulation factor Xa, and the *Bacillus subtilis* dibasic endoprotease subtilisin A [29]. Furthermore, the distribution pattern of PI8 in human tissues is different from that of furin. PI8 is mainly expressed in the nucleus of squamous epithelium of mouth, pharynx, esophagus, and epidermis, and in the epithelial layer of skin appendages, particularly in more differentiated epithelial cells [30]. Due to their different distribution patterns, PI8 seems very unlikely to interact with furin *in vivo*. As permanent inhibition against furin *in vivo* would be harmful to cells, the temporary inhibition of histone H1 [11] would be more ideal to regulate furin activity. Furthermore, histone H1 and its C-terminal part have many advantages over other inhibitors for its small size and nontoxic properties.

As pro-vWF is well known to be processed by furin [17,31,32], it was also used as a model protein to study the inhibition of furin-dependent proprotein processing by inhibitors added to the culture media, such as exogenous eglin C variants [33]. In this study, the efficacy of histone H1 in cellular inhibition of proprotein processing by furin was also shown. As described, furin can process precursor proteins in the TGN/biosynthetic and endocytic pathway, at the cell surface and possibly within the extracellular matrix following shedding. The inhibition of this processing by histone H1 added in the culture media [Fig. 1(B)] suggested that histone H1 might be involved in the regulation of furin both in the intracellular and/or in the extracellular processing events.

Our results of the co-immunoprecipitation of furin with histone H1 suggested that the interaction between histone H1 and furin possibly occurs under physiological conditions. Although we could not exclude the possibility that some of the cell disruption enables the interaction of histone H1 from lysed nuclei with furin, our data confirms that furin and histone H1 could interact with each other, possibly on the cell surface or in the extracellular matrix. The interaction in the latter could not be detected using immunofluorescence because of the lower amount of furin.

Furin is predominantly located at the TGN and also found on the cell surface, whereas histone H1 normally binds to the linker DNA and is located in the nucleus. However, it was reported that histone proteins could be located on the surface of various cells, including intestinal microvilli, monocytes and lymphocytes [13,14]. In addition, histone H1 proteins act as intestinal protein receptors for 987P fimbriae of enterotoxigenic *Escherichia coli* [34]. Histone H1 is not only located in the nucleus bound to the linker DNA, but can also be found on the cell surface and outside cells. Our results further confirm that, under physiological conditions, a portion of histone H1 exists outside cells and, under pathological conditions, histone H1 can be markedly released from cells (Fig. 4), suggesting that histone H1 most likely inhibits furin on the cell surface *in vivo*. The immunofluorescence studies further showed that furin, vWF and histone H1 could be co-localized on the cell surface. Treatment of cells with H_2O_2 markedly induces cell death and consequently the release of histone H1,

which mimics pathological conditions such as cell death induced by infection with bacteria or virus. Under these situations the released histone H1 might function as a regulator to down-regulate the activity of furin and then inhibit the maturation of bacterial exotoxins and viral glycoproteins.

Growing evidence shows that histone H1 belongs to a family of proteins with numerous biological functions [35, 36]. The proteolytic fragments of histone H1 released from epithelia possess strong antimicrobial activities [37–39] and histone H1 exerts growth inhibition in leukemia cells *in vitro*, *ex vivo* and in an animal model [40]. Furthermore, histone H1 was reported as a tumor suppressor in experimental mammary carcinoma [41]. Our results showing that histone H1 is capable of inhibiting furin might help to explain these functions, because furin plays a central role in the activation of bacterial exotoxins and in the metastasis of cancer. As a result, the inhibition of furin by histone H1 will correspondingly show antimicrobial and anticancer activity, and might represent a basic, conserved mode of post-translational control. Combining these findings we speculate that histone H1 existing outside cells or released from the cell nucleus might be involved in the regulation of furin in the proprotein processing events. We can only hope that these findings will help us better understand the complexity of furin's action in proprotein processing, as well as help us to explore the possibility of developing new therapeutic agents using furin inhibitors and regulators.

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