

Short communication

**CO-INVOLVEMENT OF THE MITOCHONDRIA AND ENDOPLASMIC  
RETICULUM IN CELL DEATH INDUCED BY THE NOVEL ER-  
TARGETED PROTEIN HAP**

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**Abstract:** HAP (a homologue of the ASY/Nogo-B protein), a novel human apoptosis-inducing protein, was found to be identical to RTN3. In an earlier study, we demonstrated that HAP localized exclusively to the endoplasmic reticulum (ER) and that its overexpression could induce cell apoptosis via a depletion of endoplasmic reticulum (ER) Ca<sup>2+</sup> stores. In this study, we show that overexpression of HAP causes the activation of caspase-12 and caspase-3. We still detected the collapse of mitochondrial membrane potential ( $\Delta\Psi_m$ ) and the release of cytochrome c in HAP-overexpressing HeLa cells. All the results indicate that both the mitochondria and the ER are involved in apoptosis caused by HAP overexpression, and suggest that HAP overexpression may initiate an ER overload response (EOR) and bring about the downstream apoptotic events.

**Key words:** Apoptosis-inducing protein HAP, Mitochondria, Endoplasmic reticulum (ER), Caspase-12, Caspase-3, Mitochondrial membrane potential ( $\Delta\Psi_m$ ), Cytochrome c

**INTRODUCTION**

Many apoptosis-related proteins have thusfar been identified and characterized. Li *et al.* [1] cloned a novel human apoptosis-inducing gene, ASY/NogoB, which encodes an endoplasmic reticulum-targeting protein without any known apoptosis-related motifs. In an earlier study, we identified and characterized another novel human apoptosis-inducing gene encoding the ASY interacting protein; it was designated HAP (a homologue of the ASY protein) [2-3]. Like ASY/NogoB, HAP also exclusively subcellularly localizes to the ER, and has no homology to other known apoptosis-related domains [1-2, 4]. Subsequently, we found that the depletion of ER Ca<sup>2+</sup> stores played the pivotal role in HAP-induced apoptotic cell death.

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The ER can function as a mobilizable calcium store that sequesters excess cytosolic calcium and acts as a reservoir for calcium signaling [5]. There is a growing body of evidence to suggest that changes in intracellular  $\text{Ca}^{2+}$  homeostasis play a critical role in the modulation of cell apoptosis [6-7]. Various cell death stimuli including growth factor withdrawal, hormonal stimulation, and drug treatment [8] are known to alter the concentration of  $\text{Ca}^{2+}$  in the cytosol, a process that plays some role in the cell death pathway. Agents that directly mobilize  $\text{Ca}^{2+}$ , e.g. the  $\text{Ca}^{2+}$ -ATPase pump inhibitor thapsigargin (TG), have been shown to trigger apoptosis in diverse cell types [9].

A collapse of mitochondrial membrane potential has been shown to occur in a variety of apoptotic model systems [10]. Induction of the mitochondrial permeability transition (MPT) has been suggested to result in the release of several apoptotic factors, including apoptotic-inducing factor (AIF) and cytochrome c [11], which initiates the intrinsic pathway of apoptosis.

Caspase-12 has been localized on the cytoplasmic side of the ER, and its processing and activation have been detected in the murine L929, B16/B16, and AKR-2B cell lines [12]. Although caspase-12 was reported not to be required for caspase-dependent ER stress-induced apoptosis [13], we speculate that only one of the ER stress-induced signaling pathways, the unfolded protein response (UPR), was described in that article. In our current study, our data revealed that HAP induced the activation of caspase-12 through another signaling pathway, the ER overload response (EOR), and eventually activated caspase-3. Moreover, HAP also induced a decrease in mitochondrial membrane potential and cytochrome c release from the mitochondria, suggesting that the mitochondria and ER were co-involved in HAP-induced apoptosis.

## METHODS

pIRES-EGFP (Clontech) and pIRES-HAP-EGFP were constructed in our laboratory [3]. The coding region sequence of the hap gene was inserted into the compatible restriction sites in the MCS of pTracerSV40 (Invitrogen) to get the recombinant plasmid pTracerhap.

Caspase-3 enzymatic activity was determined by monitoring the specific cleavage of the site-selected synthetic fluorogenic substrate Ac-DEVD-AMC and the native substrate, respectively detected using the CaspACE<sup>TM</sup> assay system and the Ac-DEVD-AMC cleavage assay. For HAP- or EGFP-transfected cells, at various time points (24, 48 and 60 h) post-transfection, cells were harvested, washed briefly with cold PBS, and then assessed according to the appropriate manufacturer's protocol.

HeLa cells transfected with pIRES-EGFP or pIRES-HAP-EGFP were harvested at several time points post-transfection, washed twice with ice-cold PBS, and lysed with modified RIPA buffer in the presence of protease inhibitors. 100  $\mu\text{g}$  of the whole cell lysate was subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. The resulting membrane

was blocked in PBS containing 5% skimmed milk, and then exposed to anti-PARP antibody. After two washes with PBS, the membrane was incubated with a goat anti-mouse antibody conjugated to peroxidase. Immunodetection was performed using the ECL Reagent.

COS-7 cells transfected with pTracerSV40 or pTracerhap were harvested and washed briefly with cold PBS at two time points (respectively at 24 and 48 h) post-transfection. Then, the cell lysates were centrifuged and resuspended in lysis buffer supplemented with protease inhibitor (0.1 mM PMSF). For each sample to be analyzed, 50  $\mu$ g of protein was resolved by 12% SDS/PAGE and transferred to a nitrocellulose membrane. Blots were blocked in a buffer with 5% dry milk, incubated with anti-caspase-12 (Oncogene), then washed and incubated with AP-coupled secondary antibody, followed by the PNPP substrate. The mitochondrial membrane potential was determined using the dye rhodamine 123 (Rh123), the uptake of which is directly related to  $\Delta\Psi_m$ . At several time points (16, 24, 36, 48 and 60 h) post-transfection, HeLa cells were harvested by trypsinization and resuspended in the medium at  $10^6$  cells/mL. The cells were incubated with Rh123 (5  $\mu$ M) for 30 min at room temperature in the dark, washed three times with PBS, and resuspended in a tissue culture medium. Then, the  $\Delta\Psi_m$  was measured by fluorescence emission on a FACScan flow cytometer (Becton Dickinson).

At different time points (16, 24, 36, 48 and 60 h) post-transfection, HeLa cells were collected and resuspended in 0.8 ml of ice-cold buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM  $MgCl_2$ , 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 0.1 mM TLCK and 0.1 mM TPCK). After 15 min on ice, the cells were homogenized in a Dounce homogenizer and centrifuged for 10 min at 750 g in order to remove unlysed cells and nuclei. The supernatant fluids were transferred to new tubes and centrifuged again. The supernatant fluids from the second centrifugation represent the cytosolic fractions, whereas the pellets, resuspended in buffer A, represent the mitochondrial fractions. The protein concentration in the mitochondrial and cytosolic fractions was determined using the Bio-Rad protein assay system. Equivalent amounts of the mitochondrial and cytosolic fractions underwent Western Blot using the antibody against cytochrome c, clone 7H8.2C12 (PharMingen). The cytochoxidase subunit IV was also detected using the rabbit antibody for each subcellular fraction.

## RESULTS AND DISCUSSION

In this study, activation of caspase-3 in cellular extracts of HAP-transfected cells (indicated as a cell population with green fluorescence) was evaluated by monitoring the cleavage of specific substrates by caspase-3 (Fig. 1A). The resulting time-dependent apoptosis in HeLa cells triggered by HAP overexpression was accompanied by a similar time-dependent activation of caspase-3, as indicated by the cleavage of the specific fluorogenic substrate Ac-DEVD-AMC. In the cellular extracts containing only EGFP-transfected cells, no

caspase-3 activation was detected, as indicated by the lack of cleavage of the specific substrate. The activation of caspase-3 was also identified by the cleavage of its native substrate PARP, a commonly used measurement of caspase-3-like enzymatic activity, which further confirmed the activation of caspase-3 in the process of HeLa cell apoptosis induced by HAP overexpression. As shown in Fig. 1B, the intact PARP (116 kDa) was cleaved to an 85 kDa signature fragment in HAP-overexpressing HeLa cells, whereas no cleavage of PARP was detected in EGFP-transfected or untransfected HeLa cells.

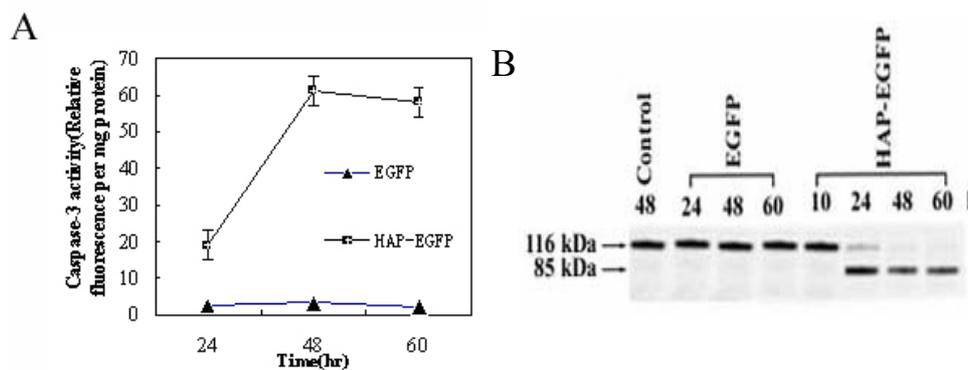


Fig. 1. Caspase-3 activation occurred in the process of HAP-induced apoptosis. A - Cell lysates were analyzed for the cleavage of the caspase-3 specific synthetic fluorogenic substrate. At 24, 48 and 60 h post-transfection, cellular lysates of HAP-overexpressing cells or EGFP-transfected cells were prepared and then analyzed by enzymatic reaction for the activation of caspase-3. The extent of substrate hydrolysis is expressed as arbitrary fluorescence units per milligram of protein. Relative fluorescence units (RFU) were calculated by subtracting the blank fluorescence from the sample fluorescence and dividing by the protein content of the sample. The data represents the averages  $\pm$  SEM from three separate experiments. B - Cell lysates were analyzed for the cleavage of the native caspase-3 substrate PARP. The intact PARP (116 kDa) was cleaved to an 85 kDa signature fragment in HAP-overexpressing cells.

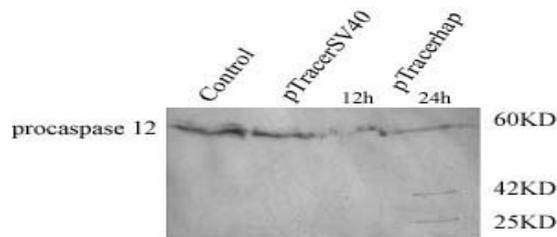


Fig. 2. Caspase-12 activation occurred in the process of HAP-induced apoptosis. The cell lysates were analyzed for the cleavage of procaspase 12. Caspase-12 was specifically activated in COS-7 cells transfected with pTracerhap at 24 and 48 h, while procaspase-12 was not activated in COS-7 cells transfected with pTracerSV40. Samples were taken at the indicated time points after transfection and assayed by Western blotting analysis using a caspase-12 specific antibody that recognizes the full-length and the active form.

To determine if caspase-12 activation is involved in HAP-induced apoptosis, caspase-12 was analysed in COS-7 cell lysates with the rabbit anti-caspase-12 polyclonal antibody. 24 h after transfection, only procaspase-12 was detected in the control and COS-7 cells transfected with pTracerSV40, while in COS-7 cells transfected with the plasmid pTracerhap, procaspase-12 was found to have been activated by being cleaved into two subunits of 42 kD and 25 kD (Fig. 2). These results suggested that the ER apoptosis pathway might be involved in the apoptosis process induced by HAP.

We used the dye Rh123 to examine the effects of HAP on the relative  $\Delta\Psi_m$  in the HeLa cell line (Fig. 3A). In cells transfected with pIRES-HAP-EGFP plasmid, there were two cell populations, respectively represented by the normal  $\Delta\Psi_m$  (right peak) and the decreased  $\Delta\Psi_m$  (left peak), suggesting that HAP induced the decrease in  $\Delta\Psi_m$  in the HeLa cell line. By contrast, in the control group transfected with pIRES-EGFP plasmid, a single cell population with normal  $\Delta\Psi_m$  was detected at several time points post-transfection, and the percentage of cells was essentially unchanged (Fig. 3B).

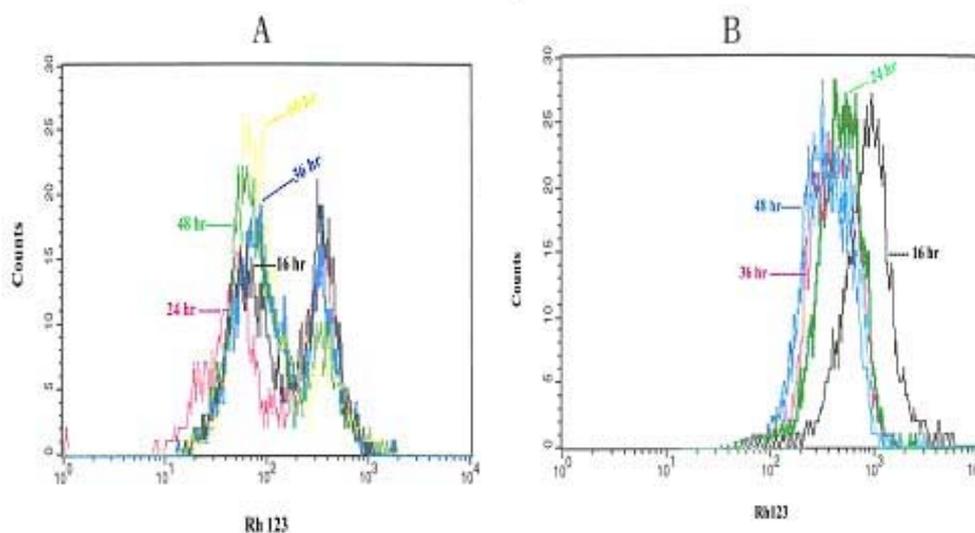


Fig. 3. HAP induced a decrease in mitochondrial membrane potential ( $\Delta\Psi_m$ ). The mitochondrial membrane potential of the control or HAP-overexpressing HeLa cells was measured using Rh123 dye. Cells with HAP overexpression (A) and the control cells (B) were assayed for changes in mitochondrial membrane potential at different time points post-transfection.

We investigated the effects of HAP overexpression on cytochrome c redistribution, and determined the presence of cytochrome c in the cytosolic and mitochondrial fractions of cells transfected or untransfected with HAP proteins (Fig. 4). Cytochrome c always resides in the mitochondrial intermembrane space, which was assessed at various times after pIRES-EGFP transfection,

except that only a small amount of cytochrome c was detected in the cytosolic fraction of cells 60 h post-transfection (Fig. 4B); this might have been caused by EGFP. However, HAP overexpression resulted in the leakage of cytochrome c from the mitochondrial fraction in a time-dependent manner, accompanied by an increase in the cytochrome c content in the cytosolic fraction (Fig. 4A). By contrast to this cytochrome c release, the inner mitochondrial membrane protein cytochrome oxidase subunit IV remained in the mitochondrial fraction in all the cell groups under all the conditions tested (Fig. 4A, B). Thus, HAP protein appears to induce cytochrome c release from the mitochondria.

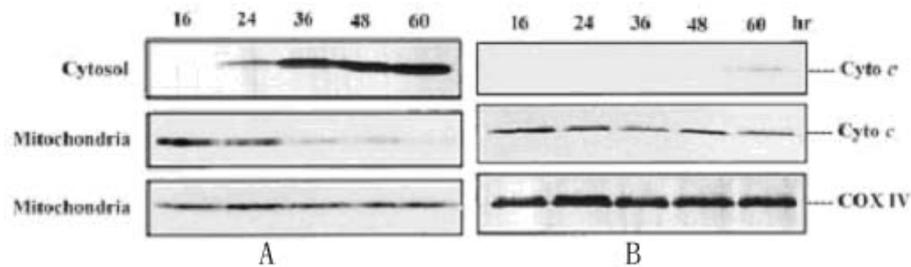


Fig. 4. The release of cytochrome c from mitochondria in HAP-overexpressing HeLa cells. Cytosolic and mitochondrial fractions were prepared for HAP-overexpressing HeLa cells (A) or HeLa cells transfected with pIRES-EGFP (B). The levels of cytochrome c were determined by Western blot analysis with anti-cytochrome c monoclonal antibody. The mitochondrial maker cytochrome oxidase subunit IV (COX IV) was used as the gel loading control.

Two ER stress-induced signal transduction pathways have been described: the unfolded protein response (UPR) and the ER overload response (EOR). The signaling pathways for UPR have been fully elucidated, while the pathway for EOR remains elusive. We only know that the ER generates two second messengers in response to this stress: calcium and reactive oxygen intermediates (ROIs) [14]. In our previous study, we revealed that over-expression of the HAP protein elicits calcium release and ROI generation (data not shown). Our current study further demonstrated that HAP induced the activation of caspase-12 in COS-7 cells, which implies that caspase-12 participates in EOR.

Cytochrome c release and  $\Delta\Psi_m$  decrease are regarded as early irreversible events during apoptosis [10, 15] related to the mitochondria. Caspase-3 plays prominent roles in the execution phase of cell apoptosis [16]. In this study, it was demonstrated that HAP overexpression induced the cytochrome c release and  $\Delta\Psi_m$  decrease. Therefore, we draw the conclusion that the apoptosis induced by HAP overexpression involves the mitochondria and endoplasmic reticulum, and that they have cross-talk in this extreme exigence. HAP is undoubtedly an efficient tool to elucidate this co-operation, particularly when other factors such as Bcl-2, ASY and CHOP are taken into account.

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