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Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors

Fabrice Chimienti[a,*], Michel Seve[a], Sandrine Richard[b], Jacques Mathieu[b], Alain Favier[a]

[a]*Laboratoire de Biologie du Stress Oxydant LRC 8M CEA associé INRA, Université J. Fourier, Domaine de la Merci, 38700 La Tronche, France*
[b]*Unité de Radiobiologie et inflammation, CRSSA, Avenue des Maquis du Grésivaudan, 38700 La Tronche, France*

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Abstract

Zinc is a potent inhibitor of apoptosis, whereas zinc depletion induces apoptosis in many cell lines. To investigate the mechanisms of zinc depletion-induced apoptosis, HeLa cells were treated with the membrane permeable metal ion chelator, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). TPEN decreased the intracellular level of zinc and induced apoptosis with a characteristic cellular pattern, i.e. cell shrinkage and formation of apoptotic bodies, with DNA fragmentation and formation of a typical DNA ladder pattern. Following TPEN treatment, caspases-3, -8, and -9 were activated and caspase target proteins, poly(ADP-ribose) polymerase, and Sp transcription factors were cleaved. These effects were inhibited by adding zinc to the medium. To assess the role of zinc in the activation of the caspase cascade, we compared zinc inhibition during tumor necrosis factor alpha/cycloheximide- and etoposide-induced apoptosis with that induced by TPEN. Zinc addition partially inhibited caspase-3 activation, but not caspase-8 and -9 cleavage in HeLa cells treated with tumor necrosis factor alpha or etoposide. These results suggest that caspase-3 is rapidly and directly activated by zinc chelation, without a requirement for an upstream event. Caspase-3 activation is therefore the main event leading to apoptosis after intracellular zinc chelation. Finally, we conclude that cellular zinc inhibits apoptosis by maintaining caspase-3 inactive.

Keywords: Apoptosis; Zinc; Caspase; Transcription factor Sp1; TPEN

1. Introduction

Apoptosis is a programmed physiological process of cell death characterized by a distinct set of morphological and biochemical changes, including cytoplasmic membrane blebbing, apoptotic body formation, nuclear condensation, and chromosomal DNA fragmentation [1,2]. Apoptosis occurs in many biological events such as embryogenesis [3] and lymphocyte selection in the thymus [4]. Furthermore, it is necessary for the development and homeostasis of all multicellular organisms, from *Caenorhabditis elegans* to humans [5]. Apoptosis can be triggered in a wide variety of cell lines by diverse stimuli, ranging from extracellular signals such as TNFα [6] to intracellular events such as an increase in the intracellular calcium concentration [7]. Moreover, during apoptosis, a sustained rise in intracellular calcium concentration is necessary to activate the calcium/magnesium-dependent endonuclease responsible for DNA fragmentation [1]. Amongst the other putative intracellular mediators of apoptosis is zinc, which may interfere with the action of calcium [8]. First, it has been shown that zinc prevents DNA fragmentation in many cell lines, in part by inhibiting the calcium/magnesium-dependent endonuclease [9]. Second, zinc chelation has been reported to induce apoptosis in different cell lines [10]. Third, recent studies indicate that zinc inhibits caspase-3, a cell death protease implicated in apoptosis [11]. Finally, changes in cellular zinc levels are sufficient to alter susceptibility to apoptosis and lead to physiopathological changes in the human body, suggesting that zinc may serve as a coordinating regulator of mitosis and apoptosis to regulate tissue growth via mo-

* Corresponding author. Tel.: +33 476 63 71 16; fax: +33 476 76 56 64.

E-mail address: Fabrice.Chimienti@ujf-grenoble.fr (F. Chimienti).

Abbreviations: Chx, cycloheximide; PARP, poly(ADP-ribose) polymerase; TNFα, tumor necrosis factor alpha; and TPEN: *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine.

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lecular targets such as endonucleases or caspase proteases [12].

Caspases (standing for cysteine-activated aspartate-directed proteases) are proteolytic enzymes activated by different pathways at the early stage of apoptosis, either through auto-activation or the proteolytic cascade [13]. Two major pathways have been described. The caspase-8 pathway mediates the TNFα or FasL extracellular signal to the mitochondria, leading to the generation of activated caspase-8, cytochrome *c* release, and pro-caspase-9 activation [14–16]. Activated caspases-8 and -9 are responsible for pro-caspase-3 and -7 activation [17]. The second pathway involves chemical compounds such as etoposide which induce apoptosis by direct activation of caspase-9 and then lead to the activation of caspase-3 [18]. The activation of caspases-3 and -7 by caspases-8 and -9 seems to be an essential step in the execution of apoptosis [19]. Caspase-3 is considered as an "executioner" of apoptosis since it is responsible for the activation of the DNA fragmentation factor, which triggers DNA fragmentation during apoptosis [20]. Caspase-3 also cleaves several proteins such as PARP [21], ICAD (inhibitor of caspase-activated DNAse), gelsolin [22], lamin [23], fodrin [24], and transcription factors [25]. Caspase-3 is inhibited *in vitro* by micromolar concentrations of zinc [11]. Moreover, changes in zinc levels induced by TPEN or zinc ionophore pyrithione can modulate caspase-3 activity in cultured cells, thereby triggering apoptosis in staurosporine-treated BE(2)-C cells or protecting them against it [26]. In contrast, TNFα induces a characteristic apoptosis in MCF-7 cells, which lack endogenous caspase-3, demonstrating that at least one other pathway exists [27,28].

We attempted herein to refine the mechanism of apoptosis induced by zinc deprivation and in particular, to define more precisely the relationship between cellular zinc concentration, caspase-3, -8, or -9 activation, and the cleavage of protein substrates such as the Sp family of transcription factors.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade from Sigma. Molecular biologicals were obtained from Promega, Amersham Pharmacia Biotech, or Roche. TNFα, Chx, and TPEN were from Sigma. Etoposide was from TEVA Pharma. Anti-Sp1 (PEP-2), anti-Sp3 (D20), anti-Sp4 (V20), anti-PARP, anti-caspase-3, anti-caspase-9, and anti-actin antibodies were purchased from Santa Cruz Biotechnology. Anti-caspase-8 antibody was from Calbiochem. Zinquin ethyl ester was purchased from TRC.

2.2. Cell culture and whole cell extract

HeLa cells were cultured in Opti-MEM (modified Eagle's medium, Life Technologies) in the presence of 5% heat-inactivated fetal bovine serum, but no antibiotics. Cells were treated with various inducers of apoptosis: 30 μM TPEN, 40 μg/mL of etoposide, or 10 ng/mL of TNFα plus 10 μg/mL of Chx, with or without 100 μM zinc sulfate. After 0, 1, 2, 3, 6, or 9 hr of treatment, 5.10^6 to 10^7 cells were scraped and centrifuged at 1000 *g* for 3 min. Cell pellets were lysed by 3 cycles of freeze/thawing. After centrifugation (12,000 *g*, 10 min, 4°), aliquots of supernatants were frozen in liquid nitrogen and stored at −80°. The protein concentration was estimated by the Pierce Protein Assay, using BSA as the standard.

2.3. Cellular zinc content determination

The intracellular zinc was estimated by using the zinc-specific flurorogenic probe zinquin-ethyl-ester. Cells were incubated with a micromolar solution of zinquin ethyl ester in PBS at 37° for 30 min. Then, cells were washed twice with PBS and observed under an inverted fluorescence microscope (excitation wavelength 365 nm, emission wavelength 420 nm). The TPEN-induced zinc depletion was followed by spectrofluorometric assay on cell suspensions (5.10^6 cells). Cells in suspension were treated as above, and the fluorescence of 2-mL portions ($2.5\ 10^6$ cells/mL) in PBS was measured at an excitation wavelength of 365 nm and emission wavelength of 480 nm in a Perkin Elmer LS 50B luminescence spectrophotometer. After stabilization of the signal, 30 μM TPEN was added and the fluorescence of the cell suspension monitored for 30 min.

2.4. Apoptosis detection and measurement

Apoptosis was evaluated using the characteristic DNA ladder pattern after electrophoresis and measurement of free nucleosomes. DNA was isolated according to a modification of Herrmann's method [29]. The DNA was stained with ethidium bromide and visualized under UV light. The measurement of the amount of free nucleosomes (mono- and oligonucleosomes) was evaluated using an ELISA kit for detection of histone-associated DNA fragments (Roche). Determination of the amount of nucleosomes in the culture supernatant was also assessed to determine the occurrence of necrosis.

2.5. Electrophoretic mobility shift assay (EMSA)

The method has been previously described [30]. The oligonucleotide probe (5′-ACGTATTCGATC**GGGGCGGGG**CGAGC and 5′-ACGTGCTCG**CCCCGCCCC**GATCGAAT) was labeled by in-filling with Klenow DNA polymerase (Roche). The probe was labeled with 3 μCi of [α-^{32}P]dATP and [α-^{32}P]dCTP and unlabeled dTTP and dGTP

(Amersham Pharmacia Biotech), purified on a Sephadex G-25 column (Roche) and stored at −20° until use.

2.6. Caspase activity measurement

The caspase assay was performed by adding a specific fluorogenic substrate to the cell lysate for 1 hr at 37° to determine caspase-3, -8, and -9 activities. The specific fluorogenic substrates used in the assay were: Ac-DEVD-AMC (acetyl-asp-glu-val-asp-7-amido-4-methyl-coumarin, Becton Dickinson) for caspase-3, Ac-IETD-AMC (acetyl-ile-glu-thr-asp-7-amido-4-methyl-coumarin, Calbiochem) for caspase-8, and Ac-LEND-AMC (acetyl-leu-glu-his-asp-7-amido-4-methyl-coumarin, Euromedex) for caspase-9. The activated caspases cleave their specific substrate, thereby releasing the fluorogenic AMC (excitation wavelength 380 nm, emission wavelength 460 nm). Specific caspase inhibitors were used to control the specificity of the enzymes for their substrates.

2.7. Western blot analysis

Typically, 30 μg of total protein was loaded on 10 to 14% SDS–PAGE, transferred onto nitrocellulose membranes, and immunoblotted with antibodies against Sp1, Sp3, Sp4, PARP, caspase-3, caspase-8, caspase-9, or actin, followed by an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The analysis of Western blot was carried out according to standard procedures using ECL (Amersham Pharmacia Biotech). Actin was used as a non-degraded control protein.

2.8. Statistics

The data were analyzed using the ANOVA method with the Newman-Keuls test.

3. Results

3.1. Induction of apoptosis by intracellular zinc depletion

Intracellular zinc was visualized by loading HeLa cells with the specific fluorescent probe zinquin ethyl ester. The direct observation of resting cells using fluorescence microscopy showed a heterogeneous distribution of zinc with a higher content in the cytoplasm compared to the nucleus. The cytoplasm was inhomogeneous and presented discrete high-intensity spots (Fig. 1A). The treatment of these cells with 30 μM of the metal chelator TPEN resulted in a very rapid decrease in intracellular fluorescence, with the fluorescence not being detectable after 20 min (Fig. 1B). This rapid decay of the fluorescence signal was confirmed by measuring the total fluorescence of an HeLa cell suspension in a spectrofluorometric assay. Following TPEN addition to the medium, the fluorescence level, and hence the zinc concentration, decreased rapidly to reach a minimum after 10 min, a level that was maintained for a further 20 min (Fig. 1C).

As previously observed after 9 hr of TPEN addition, HeLa cells presented the characteristic pattern of apoptosis [30]. During the first hours, cells began to shrink and lost their adhesion properties, then blebs appeared on membranes and the nuclei condensed. After 9 hr, the formation of apoptotic bodies occurred (Fig. 1, D and E). Following TPEN treatment, DNA fragmentation and the formation of a typical DNA ladder pattern at 6 hr confirmed the induction of apoptosis by zinc chelation (Fig. 2A). This was further corroborated by a significant increase in the intracellular concentration of free nucleosomes after 9 hr. This internucleosomal fragmentation of DNA was reversed efficiently during the first three hours following the TPEN treatment by addition of 100 μM zinc to the medium (Fig. 2B). Beyond this point, extracellular zinc had no effect and DNA fragmentation became irreversible. Taken together, these results confirmed the proapoptotic effect of zinc deprivation. According to the kinetics of TPEN-induced apoptosis, all the following experiments were done during or at 9 hr of incubation.

3.2. Zinc chelation-induced caspase-3, -8, and -9 activation

As the caspase proteins are responsible for the degradation of several target proteins during apoptosis, we examined whether they were activated during TPEN-induced apoptosis. Caspase-3, -8, and -9 activities, measured in HeLa cell extracts either by a biochemical assay or by detection of activated proteins by Western blot, were increased following TPEN treatment (Fig. 3). Caspase-3-like activity was strongly increased after 9 hr of zinc depletion, and simultaneous addition of 100 μM zinc sulfate completely inhibited this activity (Fig. 3A). Furthermore, zinc addition decreased slightly, but significantly, the basal activity of caspase-3 in control cells. The increase in caspase-3 activity was confirmed by the observation of two active fragments (p12 and p17) detected by a specific antibody and resulting from the proteolytic cleavage of pro-caspase-3 (Fig. 4A). Moreover, caspase-3 activation seems to be an early event, because the p12 and p17 fragments were detected at 1 hr after TPEN treatment. The appearance of caspase-3 p12 and p17 fragments was inhibited by zinc. Caspase-8-like activity was also strongly increased after 9 hr of zinc depletion (Fig. 3B), and this was correlated with the progressive proteolysis of pro-caspase-8 and the appearance of the intermediate p28 subunit and p18- and p10-activated caspase-8 polypeptides (Fig. 5A). The activation of caspase-8 was also inhibited by simultaneous addition of zinc (Fig. 5A). Similarly, caspase-9 was activated after 9 hr of zinc depletion, but the activation seemed to be weak compared to the activation of caspases-3 and -8 (Fig. 3C). The induction of pro-caspase-9 proteolysis by zinc chelation

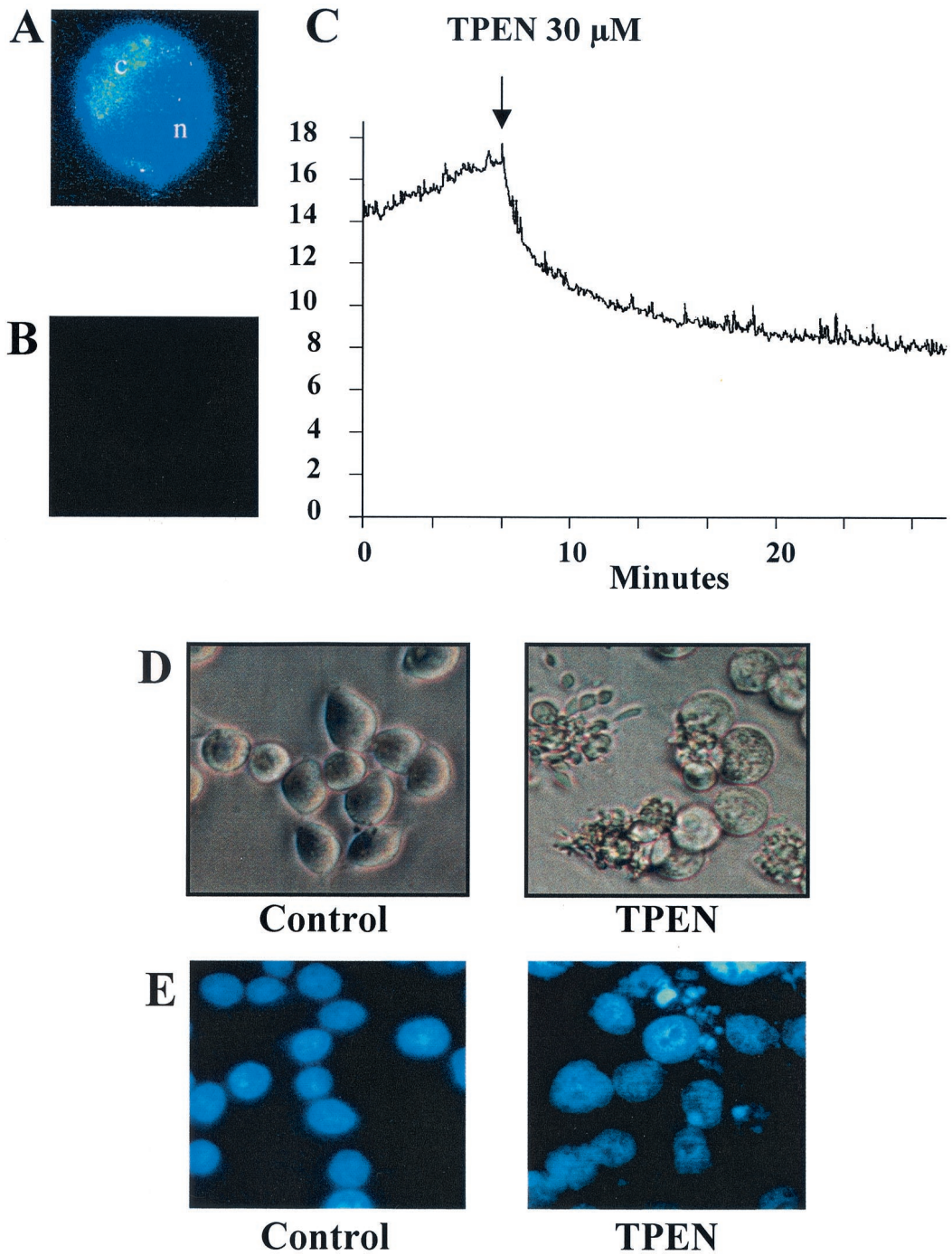

Fig. 1. Effect of TPEN on zinc content and morphology of HeLa cells. TPEN-induced intracellular zinc depletion in HeLa cells was monitored using zinquin ethyl ester as a fluorescent probe. (A) Fluorescence microscopy photography of a single HeLa cell loaded with 25 μM zinquin (n: nucleus, c: cytoplasm). (B) Fluorescence microscopy photography of the same cell, 20 min after the addition of 30 μM TPEN. (C) Spectrofluorimetric kinetics assay. (D) Phase-contrast microscopy of control HeLa cells or 9 hr after addition of 30 μM TPEN. (E) Fluorescence microscopy of control HeLa cells stained with Hoechst 33342 or 9 hr after addition of 30 μM TPEN.

was observed by Western blot, and this activation was prevented by zinc supplementation (Fig. 6A). However, the Western blot of caspase-9 following TPEN treatment suggested a late, non-specific degradation of both pro-form and activated p10 and p35 subunits of caspase-9. This degradation could explain the low level of activity measured at 9 hr by the biochemical assay.

3.3. Partial zinc inhibition of caspase-3 but not caspase-8 and -9 activation by TNFα and etoposide, respectively

To identify the target of zinc depletion, we compared the effects of TPEN on caspase activation with two classical inducers of apoptosis, TNFα and etoposide. Both TNFα and etoposide induced the activation of caspase-3 by proteolysis

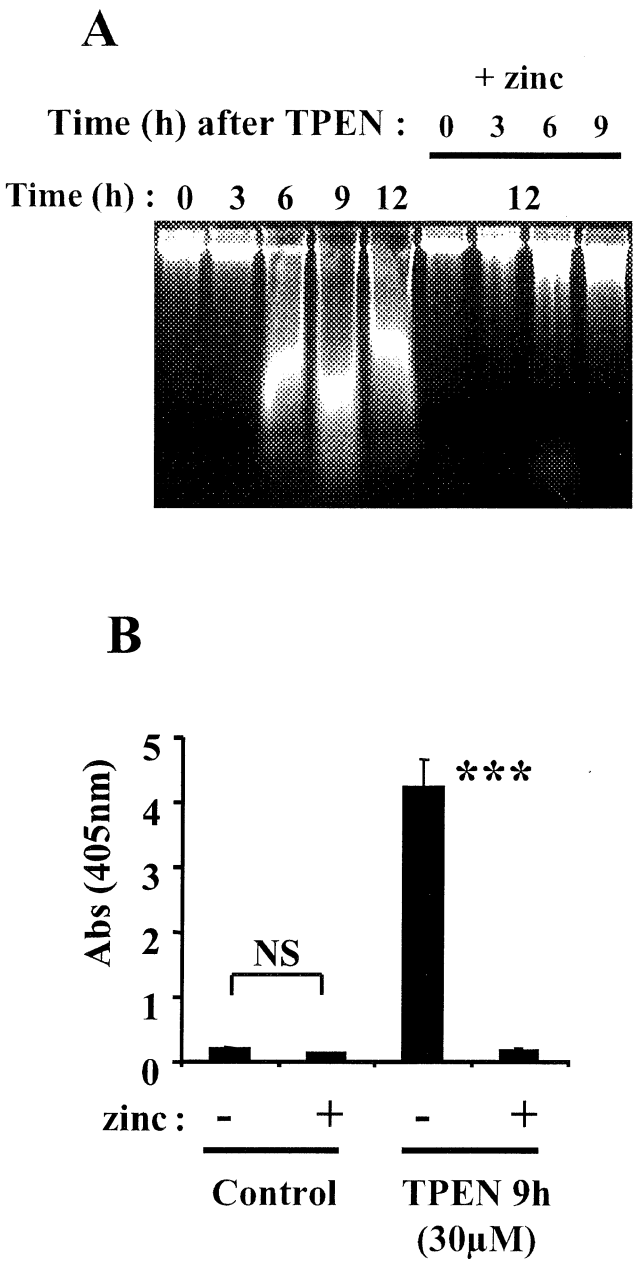

Fig. 2. Effect of TPEN on DNA fragmentation in HeLa cells. (A) Time-course of DNA fragmentation induced by TPEN. HeLa cells were treated by 100 μM TPEN for 0 to 12 hr, and 100 μM zinc was added 0 to 9 hr after TPEN as indicated. DNA fragments were separated by agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. (B) Quantification of mono- and oligonucleosome formation after 9 hr of 30-μM TPEN treatment. Bars indicate the mean ratios ± SEM. NS: not significant, ***: $P < 0.0005$.

at 3 hr (Fig. 4, B and C). The activation of caspase-3 by TNFα or etoposide was only partly inhibited by zinc addition (Figs. 3A and 4, B and C). The treatment of HeLa cells by TPEN was followed by an activation of caspase-8, which was reversible by adding zinc to the medium. TNFα also steadily activated caspase-8 with a maximum activity at 9 hr (Fig. 5B). Furthermore, unlike what was observed with TPEN, zinc failed to inhibit the TNFα-induced caspase-8 activation. Following etoposide treatment, a weak activation of caspase-8 was detected, but without a sustained activation after 6 hr (Fig. 5C).

Contrasting with TPEN treatment, caspase-9 was strongly activated by its classical inducer etoposide. This activity was sustained until 9 hr (Fig. 6C), but addition of zinc totally failed to inhibit this activation. Conversely, TNFα had little effect on caspase-9 activation, but here addition of zinc partially inhibited this activation (Fig. 6B).

3.4. Sp proteins and PARP degradation during TPEN-induced apoptosis

Several proteins, including a large number of transcription factors such as the Sp family proteins, are zinc-binding metalloproteins. The electrophoretic mobility shift assay revealed that the zinc-finger proteins Sp1 and Sp3 were affected by intracellular zinc chelation with a decrease in their binding on the consensus sequence, the GC box (Fig. 7A). This effect was inhibited by addition of zinc simultaneously with or after TPEN treatment and led to the appearance of a lower molecular weight complex when zinc was added after 9 hr of treatment (not shown). The Sp proteins from HeLa cells treated with TPEN were specifically degraded during zinc chelation-mediated apoptosis, as shown by Western blot analysis. After 9 hr, a 68-kDa proteolytic fragment was specifically recognized by an anti-Sp1 antibody. Proteolysis of Sp3 and Sp4 also occurred (Fig. 7B), and the appearance of a small peptide recognized by specific antibodies was observed (not shown). The PARP protein, a well-documented “cell death substrate”, was also degraded during this apoptosis, but at a faster rate when compared to Sp1 proteolysis.

4. Discussion

These results confirm previous studies on the proapoptotic effect of zinc depletion observed in various cell lines, including thymocytes [31], keratinocytes [32], cortical neurons [33], and peripheral blood lymphocytes [4]. We observed all the typical morphological as well as biochemical indicators of programmed cell death. The treatment of cells with TPEN at concentrations ranging from 20 to 50 μM was previously shown to induce a dose-dependent depletion of intracellular zinc [30,34], with a parallel increase in culture supernatant zinc concentration. Using a fluorescent probe, we have shown that TPEN-induced zinc deprivation is a rapid phenomenon. The intracellular level of free zinc decreases very quickly, appearing as an early event in the execution of this apoptosis.

Concomitantly to morphological signs of apoptosis, the major biochemical event observed was the activation of caspases-3, -8, and -9. Addition of an excess of zinc was described to inhibit the activation of the caspase-3 proteolytic enzyme [11]. However, we noticed herein the role of

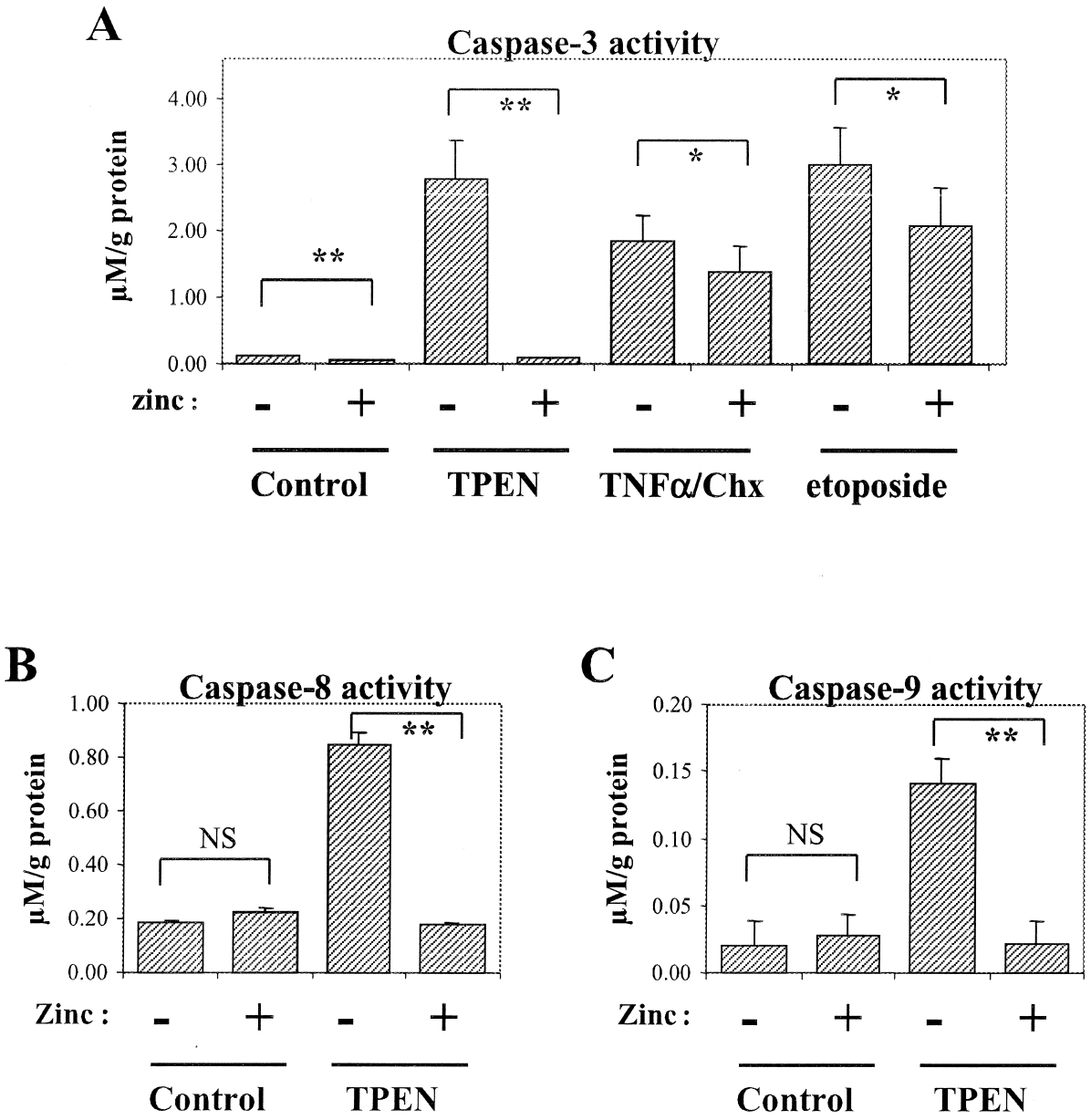

Fig. 3. Effect of TPEN on activation of caspases-3, -8, and -9. HeLa cells were treated for 9 hr with 30 μM TPEN, TNFα/Chx, or etoposide as indicated, with or without 100 μM zinc. The caspase activities were measured in whole cell lysates by cleavage of fluorescent-specific substrates for each caspase as described in Materials and Methods. Results are the mean ± SD of three experiments (NS: not significant, *: $P < 0.05$; **: $P < 0.01$).

intracellular zinc in maintaining the enzyme in the inactive form, since it was able to significantly decrease a basal activity of caspase-3 in control cells. This induction of caspase-3 activity was observed either by using an artificial fluorogenic substrate or through the cleavage of the typical cellular substrate PARP. Furthermore, MCF-7, a cell line that does not have endogenous caspase-3, is resistant to TPEN-induced apoptosis, demonstrating the strong relationship between zinc and caspase-3 activity [35]. Zinc depletion induced a strong activation of caspases-3 and -8, but a weak activation of caspase-9, suggesting that this slight increase in caspase-9 activity may result from retroactivation by other caspases, especially caspase-3. Moreover, zinc could not inhibit TNFα/Chx-induced caspase-8 activation, while it was efficient in inhibiting TPEN-induced caspase-8 activation. This suggests that caspase-8 is not directly activated by zinc chelation, but more likely through caspase-3 activation. Thus, the activation of caspase-3 is likely to be the main event leading to apoptosis after intracellular zinc chelation. The effect of zinc depletion on caspase cascades thus seems to differ from effects observed using other inducers such as FasLigand, gamma-radiation, and etoposide [36]. Caspase-3 seems to be rapidly and directly activated by zinc chelation, without any upstream event such as re-

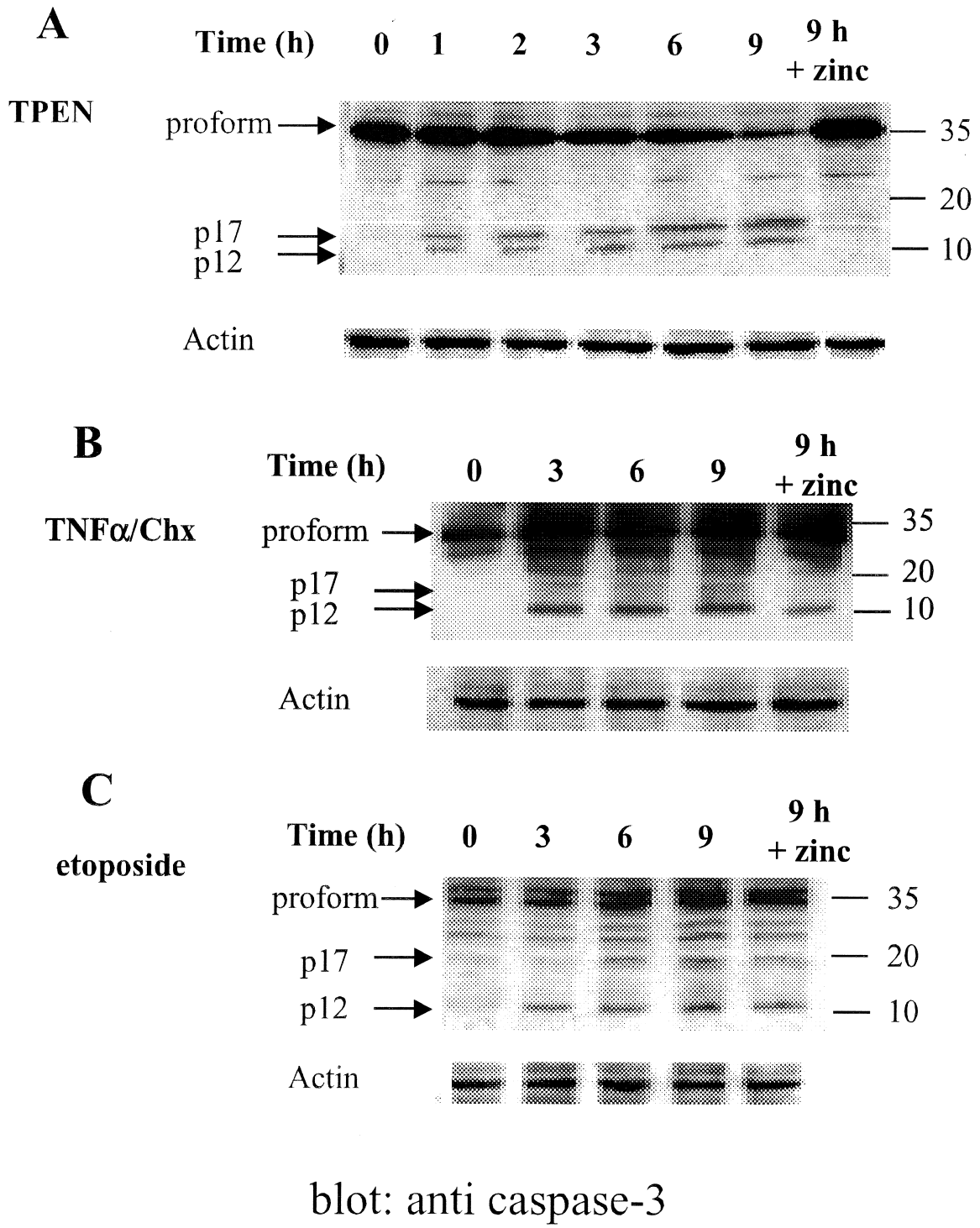

Fig. 4. Time-course of caspase-3 activation during TPEN-, TNFα/Chx-, or etoposide-induced apoptosis in HeLa cells. Activated caspase-3 was detected in whole cell lysates by Western blotting, as described in Materials and Methods. HeLa cells were treated by 30 μM TPEN (A), TNFα/Chx (B), or etoposide (C) with or without 100 μM zinc. Anti-actin Western blots were used as controls.

ceptor mediation or mitochondrial control, suggesting that zinc protects cells from apoptosis by maintaining caspase-3 inactive. Slight decreases in intracellular zinc could act as a starter of apoptosis or at least as a triggering factor amplifying the effect of various proapoptotic compounds.

Addition of an excess of zinc was able not only to inhibit apoptosis induced by zinc deficiency, but also to inhibit apoptosis induced by TNFα/Chx or etoposide (data not shown). However, zinc supplementation failed to inhibit caspase-8 and -9 activation when cells were treated with TNFα/Chx or etoposide, respectively (Figs. 5 and 6). In these cases, the inhibition of caspase-3 activity remained

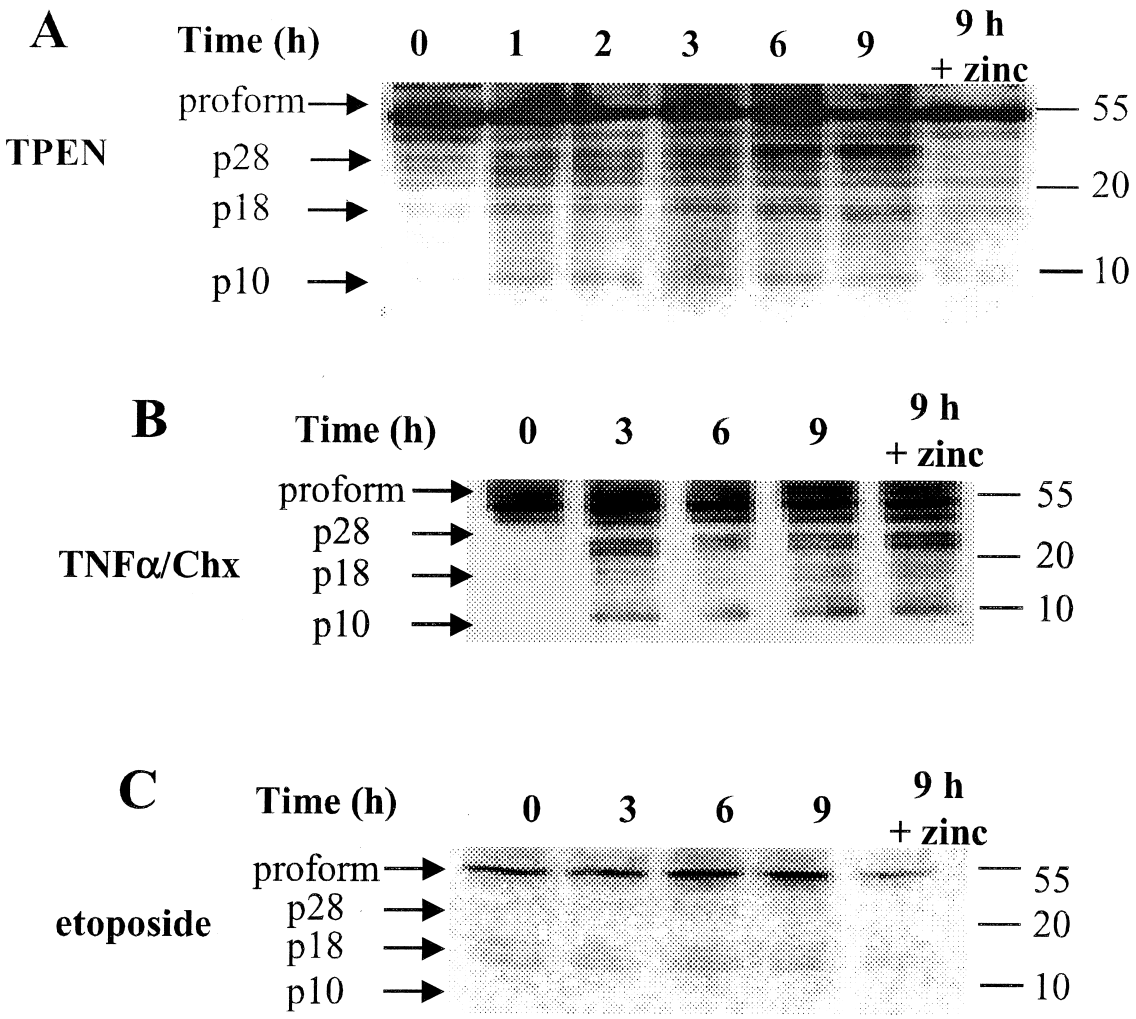

Fig. 5. Time-course of caspase-8 activation during TPEN-, TNFα/Chx-, or etoposide-induced apoptosis in HeLa cells. Activated caspase-8 was detected in whole cell lysates by Western blotting, as described in Materials and Methods. HeLa cells were treated by 30 μM TPEN (A), TNFα/Chx (B), or etoposide (C) with or without 100 μM zinc.

incomplete, compared to the inhibition observed with TPEN treatment, but was sufficient to inhibit all major signs of apoptosis, such as membrane blebbing or DNA fragmentation. Under these conditions, zinc acts as a powerful inhibitor of caspase-3 activation, as efficient as the inhibitory peptide Z-VAD-fmk [18]. Apart from the fact that zinc is an inhibitor of caspase-3, it also inhibits DNA fragmentation measured by DNA laddering and by mono- and oligonucleosome formation (Fig. 2). Zinc can therefore efficiently prevent DNA fragmentation at a late stage of apoptosis, even though it is relatively inefficient at inhibiting early events such as caspase-3 activation. This effect certainly results from the inhibitory effect described on calcium/magnesium-activated endonuclease [37]. Sp1, a zinc-finger transcription factor [38] that binds a decanucleotide sequence called the GC box, has been described as another caspase-dependent substrate in apoptosis induced by retinoic acid [25] or anti-IgM [39]. After zinc depletion-induced apoptosis, Sp1, but also Sp3 and Sp4 [40,41], two other members of the Sp transcription factor family, were cleaved (Fig. 7A). The cleavage site of Sp1 has been supposed to be located after Asp583, $ENSPD_{583}\downarrow AQPQAGR$ [39]. This location is supported by our antibody mapping, which indicated that Sp1 was cleaved after amino acid 536, Sp3 before amino acid 676, and Sp4 before amino acid 761. The cleavage of Sp proteins partly explains the decrease in DNA-binding activities observed in apoptosis ([30] and Fig. 7A). However, this decrease in binding was a very rapid

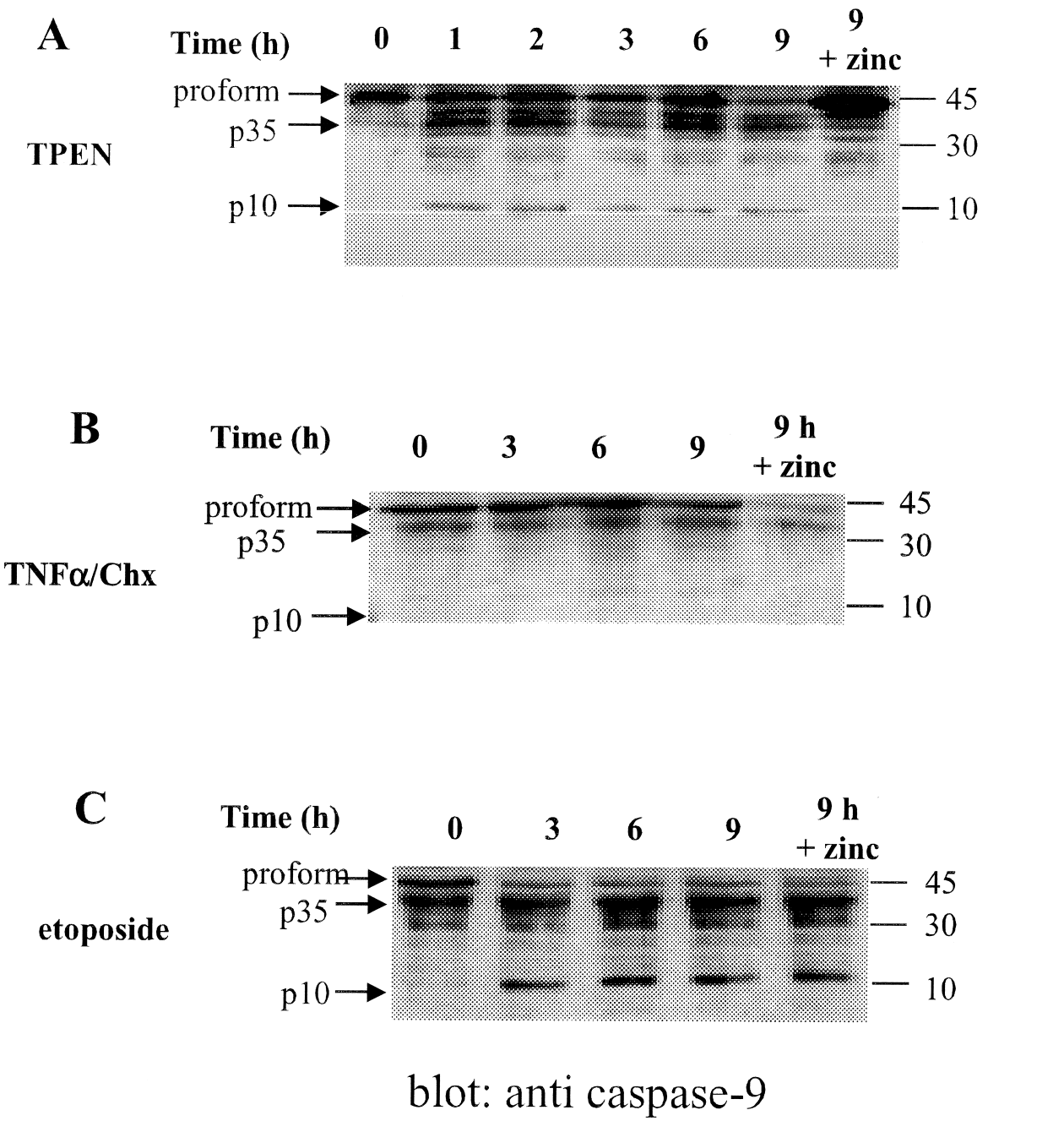

Fig. 6. Time-course of caspase-9 activation during TPEN-, TNFα/Chx-, or etoposide-induced apoptosis in HeLa cells. Activated caspase-9 was detected in whole cell lysates by Western blotting, as described in Materials and Methods. HeLa cells were treated by 30 μM TPEN (A), TNFα/Chx (B), or etoposide (C) with or without 100 μM Zinc.

phenomenon compared to cleavage by caspase activation. This rapid decrease in Sp1-binding activity could be explained by the loss of zinc from the zinc fingers of the proteins. There was also a difference in the kinetics of Sp protein proteolysis compared to the degradation of PARP that started after 3 hr of zinc depletion. The PARP was almost entirely degraded after 6 hr of TPEN treatment. The disappearance of Sp proteins was slower, beginning after 6 hr. Such a difference may have resulted from the difference in accessibility and environment of the cleavage site, leading to a lower affinity of Sp polypeptides for proteases compared to PARP. The cleavage of Sp proteins, as well as PARP, was totally reversed by the addition of zinc after TPEN treatment, demonstrating the responsibility of zinc depletion in the mechanism. Zinc addition continued to be efficient in reversing TPEN-induced Sp1 cleavage for as long as 6 hr after TPEN-induced zinc depletion in HeLa cells, at a time similar to the increase in caspase-3 activity.

The temporal relationship between zinc depletion and induction of apoptosis suggests that the modification of

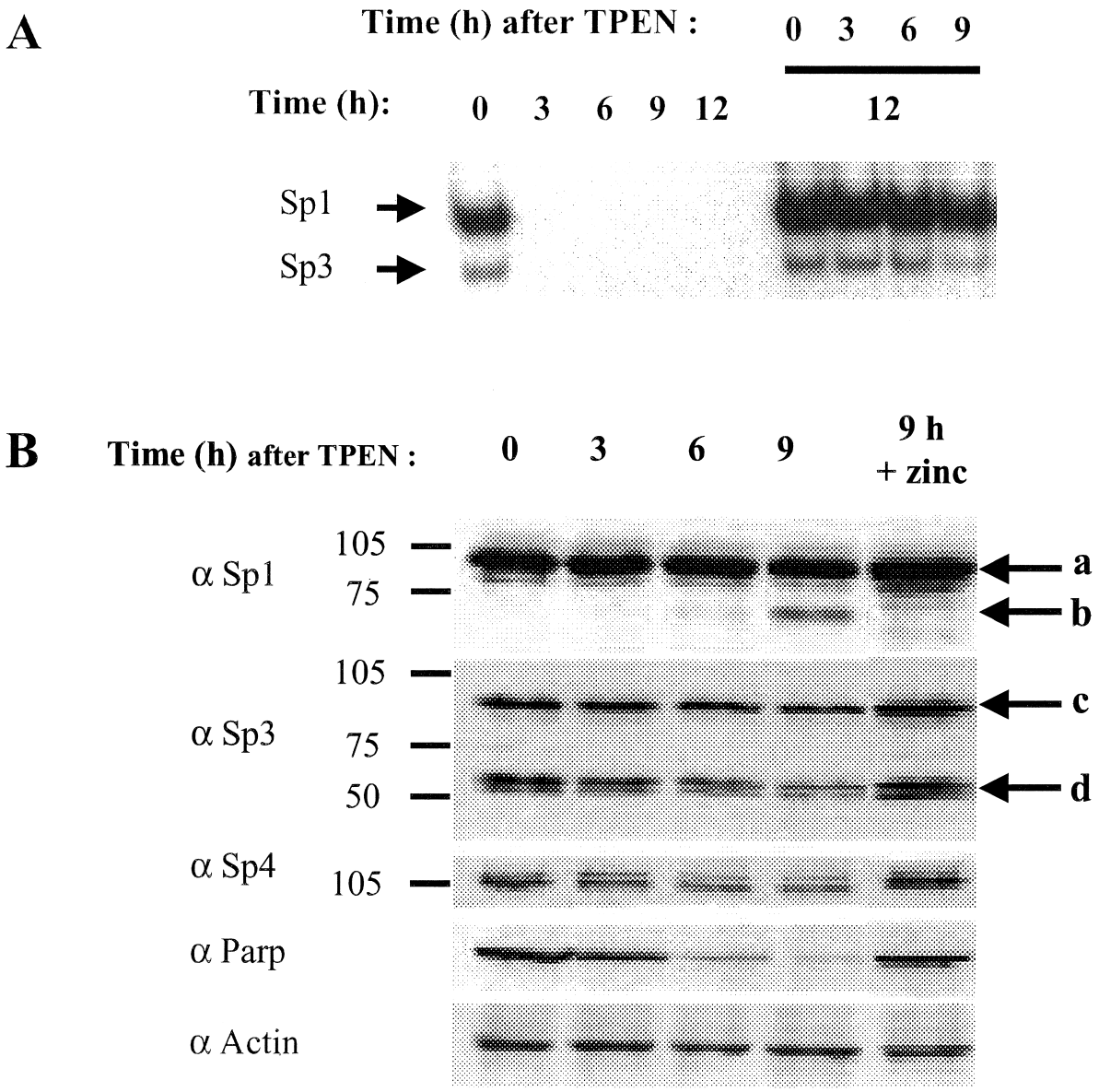

Fig. 7. Effect of TPEN on Sp family transcription factor DNA-binding activity and degradation in HeLa cells. (A) Inhibition of Sp1 and Sp3 DNA-binding activities by TPEN and reversibility by zinc. HeLa cells were treated with 30 μM TPEN for 0 to 12 hr and 100 μM zinc was added 0 to 9 hr after TPEN as indicated. Electrophoretic mobility shift assay using the ^{32}P-labeled GC box was performed on whole cell lysate. (B) Degradation of Sp proteins during TPEN-induced apoptosis and comparison with PARP proteolysis. The cleavage of Sp1, Sp3, Sp4, and PARP was analyzed by Western blot at the indicated times following treatment of HeLa cells with 30 μM TPEN in the presence or absence of 100 μM zinc. (a: Sp1 full-length, b: 68-kDa proteolytic fragment, c: 97-kDa Sp-3 polypeptide, d: 58- and 60-kDa Sp3 polypeptide). Actin was used as a non-degraded control protein.

cellular zinc could be a causal factor in switching the control of the cell cycle towards apoptosis. More globally, intracellular zinc has been presented as a regulator of apoptosis in lymphocytes [4]. Zinc has a protective effect against the colchicine-induced apoptosis of lymphocytes, which is greatly intensified by pretreatment of cells with the zinc ionophore pyrithione [31]. The level of free zinc in the nucleus is low comparative to the extracellular level. This implies a strict homeostatic control of the free zinc concentration in the cytoplasm and nucleus. Using the patch-clamp technique, calcium/zinc-permeant channels have been identified in the inner membrane of nucleus: these channels certainly contribute to nuclear zinc homeostasis [42]. From these results, we have designed a model for the regulatory role of zinc in which the latter protects cells from apoptosis by maintaining caspase-3 and calcium/magnesium endonuclease in an inactive form in the resting state of the cell. Changes in zinc concentration and/or localization in the cell constitute a major step that could initiate the cascade of events leading to cell death. Thus, a therapeutic control of zinc homeostasis in cells, using zinc chelates or modulators of zinc transporters, may be a good target for future research aimed at modulating apoptosis in diseases where zinc homeostasis is altered, such as cancer or neuronal degeneration.

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