Protease Inhibitors Induce Specific Changes in Protein Tyrosine Phosphorylation That Correlate with Inhibition of Apoptosis in Myeloid Cells

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ABSTRACT

To investigate early signaling events responsible for regulation of programmed cell death or apoptosis, we studied camptothecin (a topoisomerase I inhibitor)-mediated apoptosis in the human promyelocytic leukemia cell line HL60. We demonstrate a tight correlation between protection of HL60 cells from apoptosis-associated internucleosomal DNA fragmentation by specific protease inhibitors or protein phosphatase inhibitors, with early tyrosine phosphorylation of a single protein substrate with a molecular weight of approximately 42,000. Exposure to protease inhibitors that did not protect HL60 cells from DNA fragmentation did not result in phosphorylation of this substrate. Likewise, a protein tyrosine kinase inhibitor that did not interfere with specific phosphorylation did not prevent DNA fragmentation. Taken together, these results suggest that phosphorylation of a 42,000 substrate constitutes an important signaling event that may participate in regulation of the apoptotic response.

INTRODUCTION

Protein kinases and phosphatases form a unified system of signal transduction in the cell. Initial interactions between hormones and growth factors and their cognate receptors result in phosphorylation of specific tyrosines of their receptors. These early phosphorylations then result in a series of tyrosine and serine/threonine phosphorylations and dephosphorylations, culminating in a specific biological response (reviewed in Refs. 1 and 2). The available data suggest that signaling through protein tyrosine phosphorylation is required for induction and inhibition of apoptosis. The examples include, but are not limited to: inhibition of apoptosis in human granulocytes due to increased levels of tyrosine phosphorylation (3); requirement of tyrosine phosphorylation for apoptosis induced by ionizing radiation in human B-lymphocyte precursors (4); requirement of tyrosine phosphorylation for rescue of germinal center B cells from apoptosis (5); and activation of p34^cdk2 kinase via tyrosine dephosphorylation at the onset of CTL-mediated apoptosis (6). Although involvement of tyrosine phosphorylation in apoptosis has been widely documented, the onset of CTL-mediated apoptosis (6). Although involvement of tyrosine phosphorylation in apoptosis has been widely documented, the signaling molecules involved in the regulation of apoptosis remain largely unknown.

It has been shown recently that proteolysis also plays a central role in the apoptotic response (reviewed in Refs. 7 and 8). Various protease inhibitors interfere with apoptosis induced by diverse agents. For example, calpain inhibitors prevent dexamethasone and ionizing radiation-induced apoptosis in thymocytes and cycloheximide-induced apoptosis in granulocyte precursors (9). Serine and cysteine protease inhibitors prevent T-cell receptor complex-mediated apoptosis of activated peripheral blood T cells (10). Chymotrypsin-like protease inhibitors, chloromethyl ketones, prevent apoptosis of HL60 cells induced by cytotoxic drugs (11, 12). Interestingly, the chloromethyl ketones were also shown to inhibit ICE and ICE-like proteases (13, 14), a large family of proteases involved in regulation of programmed cell death in a number of mammalian systems (15).

If proteolytic processing and tyrosine phosphorylation are each involved in the apoptotic pathway, there may be a connection between these processes. To test for such a connection, we analyzed the pattern of tyrosine phosphorylation and the anti-apoptotic response of HL60 cells undergoing CAM-mediated apoptosis in the presence of several specific protease inhibitors or several inhibitors of cellular phosphatases. We found that protection of HL60 cells from apoptosis-associated internucleosomal DNA fragmentation by either protease or phosphatase inhibitors was tightly correlated with an early tyrosine phosphorylation of one common substrate. These results suggest a convergence of proteolytic and tyrosine phosphorylation pathways in the early events involved in the regulation of apoptosis and identify a candidate molecule that may be an important participant in this process.

MATERIALS AND METHODS

Cell Culture and Drug Treatments. Cells were maintained in macrophase serum-free medium (Life Technologies, Inc., Gaithersburg, MD). All experiments were conducted with the cells in a logarithmic phase of growth (3–5 x 10^5/ml). All drugs except OKA and herbimycin were from Sigma Chemical Co. (St. Louis, MO). OKA and herbimycin were from Calbiochem-Novabiochem Corp. (San Diego, CA). PhAsO, TPCK, CAM, and herbimycin were dissolved in DMSO to make correspondingly 1, 28, 3, and 5 mm stocks, respectively. PMSF was dissolved in methanol to make 500 mm stock. Anti-pain was dissolved in water to make 5 mg/ml stock. NEM was dissolved in ethanol to make 400 mm stock. OKA was dissolved in ethanol to make 500 mm stock. For all experiments, CAM was added at the same time as the protease inhibitors at final concentrations indicated in the figure legends. Herbimycin and phosphatase inhibitors were added 30 min prior to the addition of CAM. All incubations were carried out at 37°C, 5% CO2. For the analysis of DNA fragmentation, the cells were incubated for 5 h after the addition of CAM, and unless indicated otherwise, for 15 min after the addition of CAM for the analysis of tyrosine phosphorylation.

Analysis of DNA Fragmentation. The cells were collected by centrifugation and washed in PBS without Ca^2+ and Mg^2+; then the DNA was released from the cells as described (16). The amount of cell lysate corresponding to 3 x 10^3–1 x 10^5 cells per well, as indicated in the specific experiment, was loaded on a 1.5% agarose gel, and the electrophoresis was carried out at 2 V/cm for 17 h. Ethidium bromide-stained DNA was visualized under UV light.

Analysis of Protein Tyrosine Phosphorylation. The pattern of tyrosine phosphorylation was analyzed by antiphosphotyrosine immunoblotting as described (17) with some modifications. After completion of the incubations, the cells (2–5 x 10^5, as indicated in the specific experiment) were collected by centrifugation, washed in HBSS (Life Technologies, Inc.) containing 1 mm sodium orthovanadate, and lysed in 50 μl of SDS-sample buffer (20 mm Tris (pH 8.0), 2 mm EDTA, 2% (w/v) SDS, 20 mm DTT, 20% (v/v) glycerol, and 0.1% (w/v) bromophenol blue). The samples were boiled for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel. The proteins were electrophoretically transferred from the gel onto PVDF-Plus transfer membrane (MSI Micron Separations, Inc., Westboro, MA) and immunoblotted using a cocktail

The abbreviations used are: ICE, interleukin 1b-converting enzyme; CAM, camptothecin; OKA, okadac acid; PhAsO, phenylarsine oxide; TPCK, tosyl-L-phenylalanine chloromethyl ketone; NEM, N-ethylmaleimide; PTK, protein tyrosine kinase; MAP, mitogen-activated protein.
of two antiphosphotyrosine monoclonal antibodies (PY20, ICN ImmunoBiologics, Costa Mesa, CA; and 4G10, Upstate Biotechnology, Inc., Lake Placid, NY). Both antibodies were used at final concentrations of 400 ng/ml. The primary antibodies were detected using goat antimouse horseradish peroxidase-conjugated IgG (Sigma) and the ECL detection system (Amersham International, Buckinghamshire, England).

**Immunoprecipitations with Antiphosphotyrosine Antibodies.** Immunoprecipitations were carried out as described (18) with some modifications. After completion of the incubations, the cells (5 × 10⁶) were collected by centrifugation and resuspended in RIPA lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% (w/v) SDS, 2 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium vanadate, 1% (v/v) NP40, and 0.25% (w/v) deoxycholate]. These “total lysates” were incubated on ice for 30 min, passed through a 27-gauge needle five times to complete the cell lysis, and centrifuged at 18,000 × g for 10 min. The supernatants were precleared by continuous gentle mixing with 50 μl of RIPA-washed 50% slurry of protein A-agarose beads (Upstate Biotechnology, Inc.) for 30 min at 4°C. The “precleared lysates” were incubated for 4 h with the same cocktail of primary antibodies used for the Western blot analysis (see above; 1 μg of each antibody per sample), followed by the overnight incubation with 50 μl of 50% slurry of protein A-agarose beads. All of the incubations were carried out at 4°C with continuous gentle mixing. The immune complexes (“immunoprecipitate”) were recovered, washed, and resolved on a 12% SDS-polyacrylamide gel as described (18). The total lysate and precleared lysate were also included; each gel lane was loaded with the amount of protein corresponding to 2.5 × 10⁵ cells of the total lysate and the precleared lysate and with antiphosphotyrosine immune protein complex obtained from 5 × 10⁶ cells. The proteins were transferred to PVDF-Plus membrane and successively immunoblotted with the cocktail of antiphosphotyrosine antibodies and with rabbit polyclonal anti-rat-MAP kinase antibody (06-182; Upstate Biotechnology) as described above. After immunoblotting with antiphosphotyrosine antibodies and before immunoblotting with anti-MAP kinase antibody, the horseradish peroxidase conjugated to the antimouse IgG was inactivated with H₂O₂, as described (19).

**RESULTS**

**Effect of Protease Inhibitors on DNA Fragmentation Correlates with Their Effect on Protein Tyrosine Phosphorylation.** To investigate a relationship between proteolysis and signaling in apoptosis, we took advantage of the recent finding of Kaufmann et al. (12), who showed that different protease inhibitors protect HL60 cells from the apoptosis-associated internucleosomal DNA fragmentation induced by cytotoxic drugs. Cultures of HL60 cells were incubated with CAM in the presence or in the absence of protease inhibitors of different specificities. The following agents were used: serine protease inhibitor PMSF; serine/cysteine protease inhibitor antipain; chymotryptsin-like protease inhibitor, chloromethyl ketone TPCK; and sulfhydryl-blocking agent active against cysteine proteases, NEM (20). For each inhibitor, the pattern of DNA fragmentation and the pattern of protein tyrosine phosphorylation were analyzed. Of the protease inhibitors tested, only TPCK and NEM prevented CAM-induced internucleosomal DNA fragmentation (Fig. 1A). Interestingly, these two inhibitors were also the only ones that induced a pattern of protein tyrosine phosphorylation that included a common protein (Fig. 1B). Unlike TPCK which induced tyrosine phosphorylation of a single substrate of an approximate molecular weight of 42,000 (thereafter we will refer to it as p42), NEM induced tyrosine phosphorylation of a variety of substrates. However, the protein with a molecular weight of 42,000 was present among the many phosphorylated species induced by NEM.

To investigate whether the effect of protease inhibitors on tyrosine phosphorylation was a general one or restricted to HL60 cells, we studied the effect of TPCK on tyrosine phosphorylation in freshly isolated human monocytes from normal donors. We found that exposure to TPCK induced the appearance of an Mr 42,000 protein, and this phosphorylation correlated with inhibition of spontaneous DNA fragmentation in these cells. However, both the level of p42 and the extent of internucleosomal DNA fragmentation in native monocytes was below that observed in HL60 cells (data not shown). We also analyzed the effect of TPCK on tyrosine phosphorylation of the human erythroleukemia cell line K562. A Mr 42,000 phosphorylated protein was constitutively present in K562 cells, and phosphorylation of this protein was not enhanced by TPCK (data not shown).
Mechanism of Increased Phosphorylation of the M_42,000 Protein. The above results suggest that protease inhibitors may directly or indirectly affect the level of activity of cellular kinases and/or phosphatases that influence the level of tyrosine phosphorylation in HL60 cells. Furthermore, if tyrosine phosphorylation of the M_42,000 protein is required for prevention of apoptosis-associated DNA fragmentation in HL60 cells, PTK inhibitors might block and/or phosphatase inhibitors might mimic the effect of protease inhibitors on both tyrosine phosphorylation and internucleosomal DNA fragmentation. To test this and thus determine whether activation of kinase or inhibition of phosphatase activity was operative in this protease inhibitor-induced response, we examined the effect on HL60 cells in the presence of CAM of two phosphatase inhibitors, PhAsO and OKA, which have been implicated previously in the inhibition of apoptosis in other systems (Refs. 3 and 21; PhAsO, an inhibitor of CD45 protein tyrosine phosphatase, and other PTPases, Ref. 22; OKA is an inhibitor of serine/threonine phosphatase types 1 and 2A, Ref. 23). As shown in Fig. 2, both phosphatase inhibitors induced tyrosine phosphorylation of a substrate that co-migrated with the TPCK-induced M_42,000 protein. PhAsO treatment induced phosphorylation of numerous proteins, which included p42, whereas exposure to OKA resulted in tyrosine phosphorylation of only p42. Notably, the heterogeneous effect of PhAsO versus the specific effect of OKA parallels the heterogeneous effect of NEM versus the specific effect of TPCK on tyrosine phosphorylation (Fig. 1). Importantly, both PhAsO and OKA inhibited CAM-induced DNA fragmentation; the protective effect was more profound with PhAsO but was reproducibly present with both inhibitors.

To determine whether inhibition of PTK activity interfered with protease inhibitor-mediated tyrosine phosphorylation and DNA fragmentation, HL60 cells were exposed to CAM, TPCK, and a PTK inhibitor, herbimycin A. As shown in Fig. 3, herbimycin A was ineffective in suppressing the phosphorylation of the M_42,000 protein and in preventing the inhibitory effect of TPCK on CAM-induced DNA fragmentation. Minimal inhibition of the CAM-induced DNA fragmentation by herbimycin in the absence of TPCK is seen in Fig. 3, but it is weaker than the effect of TPCK on DNA fragmentation. Exposure to another PTK inhibitor, genistein, in the presence of CAM and TPCK was toxic to HL60 cells within the duration of the experiment and, therefore, could not be evaluated (data not shown). The above results suggest that activation of herbimycin-sensitive PTKs in HL60 cells is not responsible for the induction of tyrosine phosphorylation by TPCK.

The M_42,000 Protein Is Not MAP Kinase. MAP kinases p42\textsuperscript{ERK} (ERK-2) and p44\textsuperscript{ERK} (ERK-1) have been implicated in the regulation of a wide variety of cellular processes (24). Recently, MAP kinase activation has also been shown to prevent nerve growth factor withdrawal-induced apoptosis of the rat pheochromocytoma cell line PC-12 (25). Because of these data and because we found that the mobility of the common phosphorylated substrate induced by the protease and phosphatase inhibitors falls within the range of the mobility of the MAP kinases, we examined whether the M_42,000 protein is MAP kinase. For this experiment, we immunoprecipitated M_42,000 protein from the HL60 cell lysates using antiphosphotyrosine antibodies, fractionated the immunoprecipitated fraction on SDS-polyacrylamide gel, and confirmed that p42 was present in the immunoprecipitated fraction by Western blot analysis with antiphosphotyrosine antibodies (Fig. 4A). Total lysate and precleared lysate fractions (see “Materials and Methods”) were also included to compare the position in the gel of the immunoprecipitated p42 protein with that in the unfractionated cell lysate. After the presence of p42 protein in the immunoprecipitate was confirmed, the same blot was reacted with the anti-MAP kinase antibody (Fig. 4B). The results of this experiment show that although the p42 protein was present in the immunoprecipitated fraction (Fig. 4A), it was not detected with the anti-MAP-kinase antibody (Fig. 4B). These results suggest that the M_42,000 protein is not a MAP kinase. The uniform amount of MAP kinase detected in all samples makes it unlikely that lack of anti-MAP kinase reactivity in the immunoprecipitate was due to differences in gel loading. The presence of p44 ERK1 in the immunoprecipitate (Fig. 4B) serves as a control for the anti-MAP-kinase reactivity against the immunoprecipitated fraction.

Induction of Tyrosine Phosphorylation by TPCK Is an Early Event That Correlates with Inhibition of DNA Fragmentation. To further investigate the significance of the phosphorylation of the M_42,000 protein, we measured the concentration effect of TPCK on
The time course of TPCK-induced phosphorylation is shown in Fig. 6. The signal appeared within 1 min after the addition of TPCK, the shortest time that could be measured reliably, and was maximal after 2–3 min of exposure to TPCK. The signal diminished somewhat after 2.5 h but did not return to baseline within the duration of the experiment. Hence, the effect of TPCK on both p42 phosphorylation and DNA fragmentation was concentration dependent, time dependent, and parallel for each effect.

DISCUSSION

This study demonstrates that specific protease inhibitors, TPCK and NEM, and specific phosphatase inhibitors, PhAsO and OKA, induce an early increase in tyrosine phosphorylation of one common protein substrate in the promyelocytic leukemia cell line HL60. Moreover, this phosphorylation correlates with the protease and phosphatase inhibitor-mediated arrest of internucleosomal DNA fragmentation in HL60 cells. It has been suggested previously that in the chain of events leading to apoptosis, proteolysis and DNA fragmentation are linked, and a proteolytic step precedes and is required for apoptosis (11). One argument in favor of this hypothesis is that several low molecular weight protease inhibitors reverse the effect of cytotoxic drugs on DNA fragmentation in different systems (11–12, 26). A simple explanation of this result is that these protease inhibitors suppress the activity of a protease that induces an endonuclease responsible for internucleosomal DNA fragmentation. Our results do not disagree with this interpretation. They suggest, however, that the relationship between protease inhibitors and apoptosis is more complex than was thought previously, i.e., we found that protease inhibitors induce an early signal in HL60 cells, and that there is a tight correlation of this signal with the arrest of DNA fragmentation. This

![Diagram of protein tyrosine phosphorylation](image)

The pattern of tyrosine phosphorylation was determined by immunoprecipitation with antiphosphotyrosine antibodies and examination of the phosphorylated proteins by SDS-PAGE and autoradiography. The results of two independent experiments are shown in Fig. 5. B, analysis of protein tyrosine phosphorylation. In both experiments, HL60 cells were simultaneously exposed to CAM and various concentrations of TPCK for 5 h at 37°C. Aliquots were also removed from the cell suspensions after 15 min incubation for analysis of the pattern of tyrosine phosphorylation. In both experiments, there was a high degree of correlation between the appearance of the single phosphorylated band at Mr 42,000 and the inhibition of CAM-induced internucleosomal DNA fragmentation. In the experiment shown in Fig. 5, A and B, no inhibition of DNA fragmentation or phosphorylation of the Mr 42,000 protein was seen at 2.5 or 5 μM TPCK; the full inhibition of DNA fragmentation and the maximal phosphorylation of the Mr 42,000 protein was achieved at 30 μM TPCK. As shown in Fig. 5, C and D, some inhibition of the DNA fragmentation was observed at 10 μM TPCK (Fig. 5C); at this concentration, a weak phosphorylated band at Mr 42,000 was also apparent (Fig. 5D). Parallel effects of TPCK on DNA fragmentation and on tyrosine phosphorylation were seen through the entire concentration range of TPCK examined.

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phosphorylation was performed as in Fig. 1B, as described in the legend to Fig. 1. B and D, analysis of protein tyrosine phosphorylation in HL60 cells cultured in serum-free medium were exposed to CAM (0.5 μM) in the absence and in the presence of the increasing concentrations of TPCK. The DNA was analyzed, as described in the legend to Fig. 1B.

The finding implies that protease inhibitors may initiate a signaling cascade culminating in an anti-apoptotic response. The effect of serine/threonine phosphatase inhibitor OKA and PTPase inhibitor PhAsO on apoptosis has been studied by many investigators. The picture that emerges from these studies is complex. Both agents were shown to inhibit or activate apoptosis depending upon the system, concentration used, and the time of exposure of the cells to these drugs. For example, at concentrations below 1 μM, PhAsO induced, whereas at concentrations above 1 μM, it suppressed cell death in human eosinophils and neutrophils (3). Moreover, the suppression of cell death in that system correlated with the maximal levels of PhAsO-induced tyrosine phosphorylation. Heterogeneity of the pattern of tyrosine phosphorylation in granulocytes after PhAsO treatment did not permit the authors to identify a phosphorylated species that may be functionally important for protection of granulocytes from apoptosis. In the present system, the treatment of HL60 cells with PhAsO also resulted in a heterogeneous pattern of tyrosine phosphorylation. However, OKA and TPCK induced a specific phosphorylation response. Correlation of this specific phosphorylation with the resistance to CAM-induced internucleosomal DNA fragmentation allowed us to identify p42 as a potential mediator of inhibition of apoptosis.

OKA was shown to inhibit heat- or radiation-induced apoptosis at 500 nM in several human B-cell lymphoma cell lines; but no inhibition of apoptosis was observed below 250 nM (23). However, in other systems, OKA was shown to induce apoptosis (27). Notably, in all systems, the concentration of both PhAsO and OKA that prevented apoptosis after short-term exposure caused cytoxicity and cell death after long-term exposure. A similar time-dependent effect has been observed with protease inhibitors (12).4

The finding that the serine/threonine phosphatase inhibitor OKA increases tyrosine phosphorylation of the Mr 42,000 protein suggests that in our system, OKA exerts its effect through a signaling molecule that is upstream of an immediate phosphorylation of the p42 protein. The involvement of OKA in regulating protein tyrosine phosphorylation has been documented previously (28, 29) and has been interpreted as a consequence of inhibition by OKA of serine/threonine phosphatase activity controlling the downstream PTK and/or PTPase. The effects of OKA on tyrosine phosphorylation of p42 may exemplify a similar type of an indirect effect.

The exact mechanism by which protease inhibitors induce tyrosine phosphorylation of p42 is not clear. It is possible that TPCK and NEM act through first inactivating a cellular protease. Alternatively, TPCK and NEM may directly affect the activity of signaling molecules such as phosphatases. This scenario appears quite plausible. All dual-specific phosphatases (enzymes capable of dephosphorylating both phosphotyrosine and phosphoserine/threonine residues) contain the common active site sequence motif HCXXGXXRS (30). Mutational analyses has shown that the conserved cysteine is required for the rate-limiting formation of a thiol-phosphate intermediate with the substrate (31). When the active site serine was mutated, however (30), the formation of the intermediate was unaffected, but the rate-limiting step was changed from the intermediate formation to the intermediate breakdown. This suggests that the active site serine is required for the dissociation of the enzyme-substrate intermediate. These results demonstrate the importance of both serine and cysteine for the catalytic action of dual-specific phosphatases. Given its specificity for active site serines (20), TPCK is likely to react with dual-specific phosphatases; the reactivity of cysteine-reactive NEM against several types of phosphatases has also been demonstrated (32, 33).

Irrespective of the mechanism of action, it is a novel observation that specific protease inhibitors cause tyrosine phosphorylation of a single protein that was common to all of the agents effective in arresting the CAM-induced internucleosomal DNA fragmentation. Identification of this protein will be essential for dissecting the regulatory pathways of apoptosis in cells of monocytic origin; this work

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4 N. L. Lumelsky, unpublished observations.
is now in progress. The significance of phosphorylation of the Mr 42,000 protein for apoptosis in other cell types is not known; more cell types will need to be examined to determine the extent of this phenomenon. The finding that p42 protein was constitutively phosphorylated in K562 cells and that phosphorylation of this protein was not enhanced by TPCK may be noteworthy because K562 cells are extremely resistant to apoptosis induced by cytotoxic drugs (34). Also, as to the significance of phosphorylation of the Mr 42,000 protein for inhibition of apoptosis in other cell types, the results of Baxter and Lavin (21), and Song et al. (35) may also be of relevance. These investigators used two-dimensional gel electrophoresis to analyze the pattern of phosphorylation of cellular proteins during apoptosis induced by different agents in T- and B-cell lymphoma cell lines. They found that in both systems, apoptosis was accompanied by dephosphorylation of one protein, with an approximate Mr 40,000. Moreover, OKA prevented apoptosis in all cases and inhibited dephosphorylation of this common protein. It will be important to determine whether the phosphorylated protein induced by the protease and phosphatase inhibitors in HL60 cells and the Mr 40,000 phosphorylated protein from T- and B-lymphoma cell lines are related or identical.

It will be important to determine how the phosphorylation of the p42 protein fits into a well-conserved general machinery of programmed cell death in mammalian cells. In this respect, our finding that TPCK, but not antipain and PMSF, was able to induce the phosphorylation of the Mr 42,000 protein and to inhibit the apoptosis-associated DNA fragmentation (Fig. 1) may be of significance, because it has been shown previously that TPCK, but not antipain or PMSF, can inactivate ICE and the ICE-like family of proteases, which were proposed to play a key role in programmed cell death in mammalian cells (15). In view of this effect of TPCK on ICE and ICE-like proteases, our results may indicate that TPCK on ICE and ICE-like proteases, our results may indicate that


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