Endo-exonuclease of human leukaemic cells: evidence for a role in apoptosis

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SUMMARY

Inactive forms of endo-exonuclease, activated in vitro by treatment with trypsin, have been identified in human leukaemic CEM and MOLT-4 cells. They comprise over 95% of the total single-strand DNase activity in nuclei and are mainly bound to chromatin and the nuclear matrix. The activated enzyme had Mg²⁺(Mn²⁺)-dependent, Ca²⁺-stimulated activities with single- and double-strand DNAs and RNA (polyriboadenyllic acid) and other properties characteristic of endo-exonucleases previously described. At least twice as much inactive endo-exonuclease has also been localised in extranuclear compartments of CEM and MOLT-4 cells, 85% bound to the membranes of the endoplasmic reticulum and 15% free in the cytosol. The soluble cytosolic trypsin-activatable endo-exonuclease was immunoprecipitated by antibodies raised independently to both Neurospora and monkey CV-1 cell endo-exonucleases. The free and bound enzymes of both nuclear and extranuclear compartments also cross-reacted on immunoblots with the antibody raised to Neurospora endo-exonuclease to reveal multiple polypeptides ranging in size from 18 to 145 kDa, many of which exhibited activity on DNA gels. The major species bound to the chromatin/matrix were in the 55-63 kDa range. Limited proteolysis of the large polypeptides to those of 18 to 46 kDa accompanied spontaneous chromatin DNA fragmentation to form DNA ‘ladders’ in an isolated nuclei/cytosol system.

When the leukaemic cells were treated in culture with either etoposide or podophyllotoxin to induce apoptosis, the largest polypeptides disappeared and smaller endo-exonuclease-related polypeptides of 18 to 46 kDa were detected in the nuclear extracts. The appearance of these polypeptides also correlated with extensive chromatin DNA fragmentation. In addition, there were correlations between the depletion of the major 55-63 kDa species bound to the membranes of the endoplasmic reticulum, depletion of the extranuclear trypsin-activatable activity and the onset and extent of chromatin DNA fragmentation in both cell lines. The extranuclear 55-63 kDa species may be precursors of the chromatin/matrix bound endo-exonuclease. The results indicate that endo-exonuclease plays a role in chromatin DNA degradation in mammalian cells during apoptosis.

Key words: Nuclease, Endo-exonuclease, Apoptosis, Nucleus, Endoplasmic reticulum, Leukaemic cell, Human

INTRODUCTION

Endo-exonucleases (EEs) have been purified and characterized from Neurospora crassa (Chow and Fraser, 1983), Aspergillus nidulans (Koa et al., 1990) Saccharomyces cerevisiae (Chow and Resnick, 1987; Dake et al., 1988), Coprinus cinerus (Lu and Sakaguchi, 1991), Drosophila melanogaster (Shuai et al., 1992) and monkey CV-1 cells (Couture and Chow, 1992). Their properties and proposed roles in vivo have been recently reviewed by Fraser and Low (1993). The purified enzymes have in common a divalent metal ion-dependent single-strand (ss) specific endonuclease activity which makes breaks with 5′-P and 3′-OH termini in both DNA and RNA, and processive 5′ to 3′ exonuclease activity with double-strand (ds) DNA. However, the EEs differ in their spectra of activating metal ions, in their processivities and in other minor respects. While all these EEs are dependent on Mg²⁺ or Mn²⁺, the purified mammalian CV-1 EE is synergistically activated by Ca²⁺ (Couture and Chow, 1992). The processive exonuclease of Neurospora EE requires high Mg²⁺ (10 mM), but the endonuclease nicks and then linearizes supercoiled DNA in the presence of 0.1 mM Mg²⁺ (Chow and Fraser, 1983). Neurospora and Aspergillus EEs also generate many ss-breaks and, much less frequently, ds-breaks in linear ds-DNA both of which are prevented when the terminal 5′-phosphoryl groups are removed by pre-treatment with alkaline phosphatase (Fraser et al., 1989; Koa et al., 1990). This indicates that EE diffuses on the linear duplex DNA from the termini to make the internal breaks.

Two distinct EEs have been purified from Saccharomyces, a nuclear EE of 72 kDa (Chow and Resnick, 1987) and a mitochondrial EE of 37 kDa (Dake et al., 1988). Mutation in the RAD52 gene of Saccharomyces results in very low expression of the nuclear EE, profound deficiency in recombination and recombinational ds-break DNA repair and sensitivity to a variety of mutagens (Chow and Resnick, 1988). Mutation in the uvs-3 gene of Neurospora results in deficiencies of both nuclear and mitochondrial EEs and multiple mutagen sensitivities (Fraser and Cohen, 1983; Ramotar et al., 1987). Recently, it has been found that some radiosensitive mutants of Chinese hamster ovary and radiosensitive human ataxia telangiectasia cell lines are deficient in a 67 kDa EE polypeptide which cross-reacts with antibody raised to the monkey CV-1 EE (Liu et al., 1995). These findings, together with the resemblances of EE...
nuclease activities to those of the major recombination nuclease of *Escherichia coli* (the recBCD nuclease), indicate that nuclear EEs have a role in DNA repair, in particular in recombinational ds-break repair (Fraser, 1994).

Inactive forms of EEs have been identified mainly in the cytosols, but also in nuclei of *Neurospora* and *Aspergillus* (Fraser and Cohen, 1983; Ramotar et al., 1987; Koa et al., 1990). These include a putative precursor of 93 kDa and Koa et al., 1990). These include a putative precursor of 93 kDa and EE-inhibitor complexes which may result from limited proteolysis of the precursor (Hatahet and Fraser, 1989). The inactive forms are activated in vitro by treatment with trypsin. Antibody raised to the purified 31 kDa *Neurospora* EE was used to detect the 93 kDa precursor and to immunoprecipitate active polypeptides of 76, 66, 43, 37, 31 and 28 kDa which were detected by activity gel analysis of extracts and nuclear fractions in ssDNA gels (Ramotar et al., 1987). These observations indicate that the active polypeptides were derived from the precursor by limited proteolysis, but it was not clear to what extent the proteolysis occurred in vivo or in vitro during isolation and purification. The antibody was also used in immunooaffinity chromatography to purify the 72 kDa NUD1 gene product of *Saccharomyces* and the 65 kDa monkey CV-1 cell EE (Chow and Resnick, 1987; Couture and Chow, 1992).

Cell death by apoptosis is triggered in many cell types by cytotoxic (non-repairable) doses of ionizing radiations and chemotherapeutic agents and by various natural stimuli such as changes in hormone and growth factor status and unknown factors during the normal development of multicellular organisms (Kerr et al., 1972; Arends et al., 1990; Arends and Wyllie, 1991; Hickman, 1992; Schwartzman and Cidlowski, 1993). Among the various cellular changes which accompany the apoptotic process is activation of endogenous Ca$^{2+}$, Mg$^{2+}$-endonucleases which make ds-breaks with 5'-P and 3'-OH termini in the chromatin DNA (Wyllie, 1980; McConkey et al., 1989; Arends et al., 1990; Schwartzman and Cidlowski, 1993). These lead first to extensive nicking of chromatin DNA and then to formation of 300 kbp and 50 kbp ds-DNA fragments. In many but not all affected cell types, more extensive degrada- tion occurs subsequently, releasing oligonucleosome-sized fragments, multiples of about 200 bp ds-DNA fragments (Oberhammer et al., 1993; Cohen et al., 1994; Zhivotovsky et al., 1994a).

Several Ca$^{2+}$, Mg$^{2+}$-endonucleases, including DNase I, have been proposed as candidates for the chromatin DNA-fragmenting activity in apoptosis (Wyllie, 1980; Cohen and Duke, 1984; Gaido and Cidlowski, 1991; Ucker et al., 1992; Nikonova et al., 1993; Peitsch et al., 1993). DNase I makes the appropriate breaks in DNA with 5'-P and 3'-OH termini and can occur in inactive complexes with actin (Lazarides and Lindberg, 1974; Mannherz et al., 1975, 1982). DNase I, however, is a secreted enzyme normally confined in cells to the lumen of the endoplasmic reticulum (Polzar et al., 1993), and is not expressed in all cell types (Lacks, 1981). Signifi- cantly, unlike the endogenous nuclear enzyme, DNase I does not generate the characteristic oligonucleosome-sized ds-DNA fragments when it is used in vitro to treat chromatin but rather generates random-sized DNA fragments (Machaca and Compton, 1993). Transfection and over-expression of the DNase I gene in COS cells induced an apoptosis-like chromatin DNA fragmentation and was cytotoxic when expressed in *E. coli* (Polzar et al., 1993). However, it is likely that the expression of any nuclease which nicks or causes ds-breaks in chromatin DNA can activate the endogenous nuclease to trigger apoptosis. Thus, despite acceptance (Stewart, 1994) or tentative acceptance (Walker et al., 1995) in recent reviews, it now seems unlikely that DNase I is an apoptosis nuclease (see also Discussion).

One of the nuclear Ca$^{2+}$, Mg$^{2+}$-endonucleases was shown to be a rapidly turning-over enzyme (McConkey et al., 1990; Nikonova et al., 1993). However, no inactive forms of this or other Ca$^{2+}$, Mg$^{2+}$-endonucleases have been identified. Different investigators have isolated different forms of this activity associated with polypeptides ranging in size from 18 kDa to 130 kDa (Arends and Wyllie, 1991; Caron-Leesli et al., 1991; Gaido and Cidlowski, 1991; Ucker et al., 1992; Nikonova et al., 1993; Ribeiro and Carson, 1993; Shikokawa et al., 1994). Recently, the smallest of these, NUC18, has been reported to be associated with cyclophilins, small ubiquitous cyclosporin A-binding proteins (Montague et al., 1994).

The present work provides evidence that endo-exonuclease is a prime candidate for the nuclease which fragments chromatin DNA of human leukaemic cells in apoptosis and may be the progenitor, through limited proteolysis, of multiple forms of EE similar to the various Ca$^{2+}$, Mg$^{2+}$-endonucleases that have been described in other cell lines.

**MATERIALS AND METHODS**

**Materials**

Calf thymus DNA (type I), trypsin (type III) and soy bean trypsin inhibitor (type I-S) and, except where noted, drugs used for inducing apoptosis and other chemicals were obtained from the Sigma Chemical Company (Castle Hill, NSW, Australia). Phenylmethyl-sulphonyl fluoride (PMSF) was from Boehringer Mannheim (Castle Hill, NSW, Australia). Leupeptin and pepstatin A were from AUSPEP (Parkville, Victoria, Australia). Analar Na$_2$EDTA was obtained from BDH (Kilsyth, Victoria, Australia). Tris base was from ICN (Seven Hills, NSW, Australia). SYBR Green II, a fluorescent stain for nucleic acids, was from Molecular Probes, Inc. (Eugene, Oregon). SeeBlue pre-stained protein molecular mass markers were obtained from Novex (French’s Forest, NSW, Australia). DEAE-Sepharose CL6B was obtained from Pharmacia LBK Biotechnology (North Ryde, NSW, Australia). Cell culture materials were from BRL Life Technologies (Glen Waverley, Victoria, Australia).

Antibody to the purified 31 kDa endo-exonuclease of *Neurospora crassa* was raised in rabbits (Chow and Fraser, 1983). Its specificity for endo-exonucleases was described by Fraser et al. (1986). It is relevant to this work that the antibody did not show detectable cross-reaction with bovine pancreatic DNase I. The antiserum was stored at −20°C. Before use, the IgG fraction was precipitated with 50% (NH$_4$)$_2$SO$_4$, dissolved in PBS and purified from the antiserum by chromatography on Protein A-Sepharose according to standard proce- dures. Antibody to the purified monkey CV-1 cell endo-exonuclease (Couture and Chow, 1992) raised in rabbits and supplied as a 50% (NH$_4$)$_2$SO$_4$ precipitate of the antiserum was a gift from T. Y.-K. Chow. The sample was dialysed before use to remove the (NH$_4$)$_2$SO$_4$.

**Cell culture and drug treatments**

The human T-cell leukaemic line, CCRF-CEM, was maintained in suspension culture at 37°C in RPMI 1640 supplemented with 10% foetal calf serum, 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.11% NaHCO$_3$ and 2 mM L-glutamine. Except where indicated, CEM cells were passaged from stocks for no more than three months in order to maintain them in the diploid state.
Further passages led to the accumulation of a tetraploid population (Pitman et al., 1993). The tetraploid human T-cell leukemic MOLT-4 line, obtained from L. Huschtscha, University of Sydney, was maintained in the same medium. PreB Namalwa cells were generous gifts from Drs P. Nagley and F. Vaillant, Dept of Biochemistry, Monash University, Melbourne. Two Namalwa lines were made available, the parental line (p) and one selected by growth in the presence of ethidium bromide which lacked detectable mitochondrial DNA (p0).

CEM and MOLT-4 cells were seeded at 1 to 2x10⁵ cells/ml and were treated with apoptosis-inducing drugs at 4 to 6x10⁵ cells/ml. Apoptosis was induced by treating cultures either with a 20 mg/ml solution of etoposide (VP-16) in dimethyl sulfoxide (DMSO), or with a 10 mg/ml solution of podophyllotoxin (PODO) in DMSO at the doses indicated. In each case, control cultures were treated with an equivalent concentration of DMSO. Cell membrane integrity was confirmed using the trypan blue dye exclusion test.

Assessment of apoptosis

Apoptosis in drug-treated cell cultures was assessed by morphological changes in the cells using light microscopy according to the criteria of Arends and Wyllie (1991), by flow cytometry and by agarose gel electrophoresis of DNA isolated from the cells. For assessment of morphological changes, cytofiltration preparations were made, fixed and stained with Wright’s solution using standard procedures. Flow cytometry was performed on a BD FACScan flow cytometer (Becton Dickinson) with LYSII software for analysis. The cells were fixed in ice-cold 70% ethanol and washed with standard phosphate buffered saline (PBS) before staining with propidium iodide (PI) for DNA analysis using standard techniques. PI fluorescence was collected at 564-606 nm. Ten thousand cells were examined in both control and drug-treated samples. For detection of DNA ladderung, DNA was extracted and purified according to standard procedures (Ireland and Pittman, 1995) from 1x10⁵ cells and subjected to electrophoresis in 1.8% agarose gels containing 2 μg/ml ethidium bromide. Gels were photographed using UV transillumination.

Isolation and extraction of nuclei

Nuclei were prepared routinely from 5x10⁶ CEM or MOLT-4 cells by the following procedure which was scaled up or down for different purposes. Cells were harvested at room temperature by centrifugation at 700 g for 5 minutes and then washed twice with 20 ml cold PBS. All subsequent procedures were performed at 4°C. The washed cells were lysed by resuspending in 80 ml lysis buffer (50 mM Tris-HCl, 12.5 mM KCl, 12.5 mM NaCl, 5 mM MgCl₂, 0.001% NP-40, pH 7.5, containing protease inhibitors) and incubating for 15 minutes. To ensure complete lysis, the suspension was forced through a 25 gauge needle. The lysate was then centrifuged at 700 g for 10 minutes to sediment the nuclei. The recovery of nuclei in each case was greater than 95%.

Serial salt extractions of nuclei were made in 20 mM Tris-HCl containing 5 mM Na₂EDTA, pH 7.5 (TE buffer) or TE buffer containing increasing concentrations of NaCl up to 2 M NaCl as indicated. Nuclei were incubated successively in each buffer and centrifuged at 3,400 g for 10 minutes. The viscous suspension that developed in TE buffer containing 2 M NaCl was sonicated before centrifugation to recover the insoluble nuclear matrix (pellet). The nuclear matrix material was then washed twice with TE buffer and finely resuspended in TE buffer by sonication for 15 seconds.

Protease inhibitors were included at each stage of purification and extraction of the nuclei at final concentrations of 1 mM PMSF, 10 μg/ml each of leupeptin and pepstatin A and, except where indicated, 4 μM 3,4-dichloroisocoumarin (DCIC). The inhibitors were diluted 100-fold from stock solutions in DMSO prepared fresh for each experiment.

Preparation of extranuclear fractions

CEM and MOLT-4 cells were harvested, washed twice with PBS, and either used fresh or were stored frozen at –70°C as cell pellets and thawed just prior to preparation of extranuclear fractions. Freezing and thawing of the cells up to at least one month after collection did not affect the patterns of EE polypeptides or the EE activities detected. The cells were resuspended at concentrations ranging from 0.25 to 1.0x10⁶ cells/ml in cold TE buffer containing the protease inhibitors as described above. Cell suspensions were sonicated on ice at full power in two 15 second bursts to lyse the cells. Lysates were centrifuged at 10,000 g for 10 minutes. The lysate supernatant, corresponding to a mixture of endoplasmic reticulum (ER) and cytosol (ER-cytosol) was recovered for protein determination, nuclease assays and immunoblots. For experiments designed to characterise this fraction further, fresh lysate supernatants were centrifuged at 200,000 g for 1 hour in the cold to separate the ER membranes (pellet) and this cytosol (supernatant). The ER fraction was resuspended by brief sonication in cold TE buffer.

Assays of nuclease activities and protein

Nuclease assays were routinely performed with 400 mg/ml heat-denatured calf thymus DNA (ss-DNA) as substrate or, where indicated, with 300 mg/ml polyriboadenylc acid (poly(A)). Incubations of 800 μl containing 50 mM Tris-HCl, 4 mM MgCl₂, 5 mM NaCl, 0.1 mM Na₂EDTA (pH 7.5), substrate and enzyme were carried out for either 0 minutes (blank) or 15 minutes at 37°C and the reactions stopped by the addition of 800 μl cold 0.3 N perchloric acid (PCA). After sitting on ice for at least 10 minutes, the acid-insoluble material was sedimented and 1.0 ml of supernatant removed for determination of absorbance at 260 nm. One unit of nuclease activity is defined as the amount of enzyme that releases 1 n mole of acid-soluble nucleotides per minute under the conditions of assay. The rate of the reaction was linear for at least 15 minutes. Although not a very sensitive assay, the method avoids many of the problems encountered from the presence of endogenous DNA in crude nuclear fractions with the more sensitive assays using plasmid or radiolabelled DNAs. It should be noted in addition that the conditions of assay employed were deliberately designed to minimize the activities of DNase II, mitochondrial nuclease and of DNases which are strictly Ca²⁺- dependent.

The BCA protein assay kit from Pierce was used for the determination of protein using bovine serum albumin to generate a standard curve.

Detection of polypeptides with DNase activities

Active polypeptides were detected in cell fractions and partially purified fractions after electrophoresis and renaturation in SDS-polyacrylamide gels containing 10 μg/ml ss- or ds-DNA (activity gels) by the method of Chow and Fraser (1983). The gels were washed four times for 120 minutes each with 250 ml 40 mM Tris-HCl containing 2 mM Na₂EDTA and 0.1% Triton X-100, pH 7.5, to remove SDS and then reactivated in excess 40 mM Tris-HCl containing 5 mM MgCl₂ and 2 mM CaCl₂ or as indicated for a minimum of 16 hours at room temperature. Positive control lanes in the gels contained either 8 U pancreatic DNase I or 0.1 U of Micrococcal nuclease to ensure proper renaturation. The bands of DNA digestion were visualized by staining the gels with a 1:10,000 dilution of stock SYBR Green II in water. The stained gels were photographed by UV transillumination.

Detection of endo-exonuclease polypeptides by immunoblotting

Denatured proteins (routinely 100 μg protein per lane) were run on 10% SDS-polyacrylamide gels at 70 V for 15 hours. Equal loading of lanes was checked by Coomassie blue staining of duplicate gels. The proteins were electrotransferred to nitrocellulose as described by Towbin et al. (1979). After transfer, the membranes were blocked for 60 minutes at room temperature with 5% skim milk in TBST buffer (10 mM Tris-HCl buffer, 150 mM NaCl, 0.1% Tween-20, pH 7.4). The membranes were then incubated with 0.5% skim milk in TBST buffer.
buffer containing a 1:500 dilution of the primary antibody for a further 60 minutes at room temperature, washed twice with TBST, re-blocked for 30 minutes and then incubated with a 1:7,000 dilution of the second antibody in 0.3% skim milk-TBST buffer. Finally, the membranes were washed 4 times with TBST buffer. The primary antibody used was the IgG fraction of antisemur raised in rabbits to Neurospora endo-exonuclease (see Materials and Methods). Immunoreactive polypeptides were detected with the Boehringer Mannheim Western Blotting Kit containing a peroxidase-labelled anti-rabbit antibody using the chemiluminescent reaction with luminol according to the manufacturer’s instructions.

RESULTS

The nuclease activity in leukaemic cell nuclei and ER-cytosol fractions

Preliminary characterisation of the nuclease activity in nuclei of tetraploid CEM and MOLT-4 cells (Fraser et al., 1993) indicated that it strongly resembled the major activity of Neurospora nuclei (Ramotar et al., 1987). As for Neurospora nuclei, the activities with ss-DNA, ds-DNA and poly rA were present, respectively, in ratios 2:1:1 and were found to be Mg²⁺- or Mn²⁺-dependent and optimal in the pH range 7 to 8. No detectable activity was observed in the presence of EDTA and the absence of divalent metal ion (Fraser et al., 1993). Interestingly, the three activities in crude nuclear fractions were inhibited when Ca²⁺ was included in the assay in addition to Mg²⁺.

As for Neurospora EE (Fraser and Low, 1993), the DNase and poly rAase activities of the CEM nuclear fractions were completely inhibited by 2 mM Zn²⁺ and by 25 μg/ml aurin tri-carboxylic acid (Fraser et al., 1993). At least ten times this concentration of ATA was required to completely inhibit pancreatic DNase I (data not shown). A further striking similarity between the nuclease activities of CEM and Neurospora nuclei was that the DNase and poly rAase activities were activated 2.2-fold on average by pre-treating the nuclear extracts with appropriate concentrations of trypsin (cf Ramotar et al., 1987). In addition, up to 70% of the ss-DNase activity in nuclear extracts bound to an affinity column prepared by cross-linking antibody to Neurospora EE to Protein A-Sepharose (Fraser et al., 1993), indicating cross-reaction of the mammalian activity with Neurospora EE.

Preliminary experiments were also carried out with CEM cells to test whether or not the nuclear EE might be involved in the chromatin DNA degradation which accompanies apoptosis (Fraser et al., 1993). The cells were pre-treated in culture with drugs at doses known to induce apoptosis in mammalian thymus and human leukaemic cells, 1 μM dexamethasone (DEX) for 48 hours and 17 μM etoposide (VP-16) for 3 hours (Wyllie, 1980; Kaufmann, 1989; Walker et al., 1991; Roy et al., 1992; Bicknell et al., 1994). The results indicated that the nuclear EE responded to both drug treatments that induced apoptosis, but the response was to reduce the level of active enzyme.

In the present work, much greater recovery of trypsin-activatable activity has been observed when trypsin activation of either nuclear or extranuclear fractions was carried out after pre-incubation with an excess concentration of divalent metal ion (Mg²⁺, Ca²⁺ or Mn²⁺) over the EDTA present. For example, for a TE buffer extract of CEM nuclei (nucleoplasm), containing 5 mM EDTA, the level of trypsin activation of ss-DNase activity increased from 3.9-fold in the absence of added divalent ion, to a maximum of 21-fold when the pre-incubation was carried out with a 1 mM excess Mn²⁺ over EDTA. Trypsin activations have thus been routinely used to assay the inactive ss-DNase after 10-30 minute pre-incubations with 6 mM Mn²⁺. The activations of ss-DNase and poly rAase activities in both nuclear sonicates and ER-cytosol fractions, although assessed only twice for each fraction, occurred in a ratio of 2:1 under these conditions (data not shown). This ratio is characteristic of that for the activations of the inactive forms of EE in Neurospora (Chow and Fraser, 1983; Fraser et al., 1986; Ramotar et al., 1987).

The contents of active and total EE after trypsin activation in sonicates of nuclei of diploid CEM and tetraploid MOLT-4 cells are shown in Table 1. The nuclei of the two cell lines contain similar amounts of total EE, respectively 28.0 and 23.5 units/10⁶ cells, but when account is taken of the ploidy difference, the level in MOLT-4 nuclei is about 40% of that in CEM nuclei. Previously it was noted that the nuclear content of EE in tetraploid CEM cells was double that of nuclei of diploid CEM cells (Fraser et al., 1993). It is not known whether pre-incubation with a divalent metal ion before trypsin activation is sufficient to unmask all of the latent EE present in nuclei. Sonication of the nuclei led to some activation of the latent EE since less than half the amount of active ss-DNase activity was recovered on gentle serial salt extraction (see below). As expected, the trypsin-activated EE of both cell lines was completely inhibited in the absence of added divalent metal ion, in the presence of excess EDTA and by 25 μg/ml ATA in the presence of 4 mM Mg²⁺ (data not shown).

The total EE observed after trypsin activation of the ER-cytosol fraction of the diploid CEM cells was 2.0 times higher than the total EE in CEM cell nuclei (Table 1), while the level found in the ER-cytosol fraction of the tetraploid MOLT-4 cells was 3.9 times higher than that found in MOLT-4 nuclei. Active EE was routinely found in the ER-cytosol fraction, usually about 17% of the total EE. After high speed centrifugation of the ER-cytosol, about 85% of the total EE sedimented with the ER membrane fraction. This masked activity was accessible to trypsin activation without solubilisation by Triton X-100, indicating that it is present on the surface of the ER membranes. These results indicate that most of the total ER-cytosol ss-DNase is EE in masked form, mainly associated with the ER membranes. This activity was also Mg²⁺-dependent and ATA-sensitive.

The cytosolic trypsin-activatable activity (15% of the total ER-cytosol ss-DNase) bound to DEAE-Sepharose in TE buffer and eluted free of active enzyme as a single component in a

Table 1. Single-strand DNase activities in sonicates of human leukaemic cell nuclei and ER-cytosols

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>ss-DNase activity (nmoles/minute per 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>Trypsin activation*</td>
</tr>
<tr>
<td>CEM nuclei</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>CEM ER-cytosol</td>
<td>8.7 ± 2.1</td>
</tr>
<tr>
<td>MOLT-4 nuclei</td>
<td>3.9 ± 2.2</td>
</tr>
<tr>
<td>MOLT-4 ER-cytosol</td>
<td>16 ± 4.3</td>
</tr>
</tbody>
</table>

*Total ss-DNase activity ± s.e.m. after trypsin activation.
Table 2. Immunoprecipitation of the soluble trypsin-activatable endo-exonuclease in CEM cell cytosol

<table>
<thead>
<tr>
<th>Additions</th>
<th>ss-DNase activity (Units/ml)</th>
<th>Active</th>
<th>Total</th>
<th>Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>NcEE</td>
<td>Ab&lt;sub&gt;NcEE&lt;/sub&gt;</td>
<td>Ab&lt;sub&gt;CV-1EE&lt;/sub&gt;</td>
<td>ProtA-S</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.74</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.37</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.90</td>
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<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.11</td>
</tr>
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<td>+</td>
<td>+</td>
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<td>4.28</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>0.22</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>2.58</td>
</tr>
</tbody>
</table>

linear NaCl gradient at about 0.4 M NaCl. This partially (10-fold) purified cytosolic EE also showed trypsin activation of Mg<sup>2+</sup>-dependent ss-DNase and poly rAse activities in a ratio of 2:1 (data not shown). Thus, the trypsin-activatable ss-DNase activities associated with both the ER and cytosol fractions are also characteristic of EE.

EE-related polypeptides in nuclear and ER-cytosol fractions

Immunoprecipitations of the soluble cytosolic trypsin-activatable endo-exonuclease of CEM cells by antibodies raised independently to the *Neurospora* and monkey CV-1 cell endo-exonucleases are shown in Table 2. Aliquots of cytosol were pre-incubated in the absence or presence of purified *Neurospora* endo-exonuclease, added as a positive control, without and with the indicated IgG for two hours at room temperature in TE buffer containing protease inhibitors. The mixtures were then made 0.2 M in NaCl and added to Protein A-Sepharose that had been swollen in TE buffer and washed twice in TE buffer containing protease inhibitors. The NaCl was added to reduce non-specific adsorption of the nucleases on the Protein A-Sepharose. The mixtures were then rocked in the cold for one hour, briefly centrifuged to pellet the Protein A-Sepharose and the supernatants removed and assayed for active and trypsin-activatable ss-DNase. The data show complete removal of the trypsin-activatable endo-exonuclease from the CEM cytosol by the antibody raised to the *Neurospora* enzyme and near complete removal by antibody raised to the CV-1 cell enzyme. The added *Neurospora* endo-exonuclease was also completely removed by the antibody raised to that enzyme, but in its presence the human trypsin-activatable endo-exonuclease was not completely removed. This indicated the the *Neurospora* enzyme competed effectively with the human enzyme for reaction with that antibody. In contrast, the antibody to the CV-1 cell endo-exonuclease did not immunoprecipitate the *Neurospora* enzyme, but removed human trypsin-activatable enzyme. When the cytosols containing the *Neurospora* endo-exonuclease were treated with trypsin, some loss of ss-DNase activity of the *Neurospora* enzyme was observed. This is the result of the sensitivity of its exonuclease activity to trypsin treatment (Chow and Fraser, 1983).

ImmunobLOTS of serial salt extracts of CEM nuclei probed with antibody raised to the purified EE of *Neurospora*. The extracts were prepared with (A) and without (B) 3,4-dichloroisocoumarin (DCIC) in the cocktail of protease inhibitors used in the TE buffer mix for extraction. The TE buffer contained either 0, 0.1, 0.2, 0.3, 0.4 or 0.5 M NaCl. A nuclear sonicate (SON) served as a control. The nuclear matrix fraction (MAT), obtained after extraction with TE buffer containing 2.0 M NaCl, is also shown in B. The lanes were equally loaded with 100 μg protein. The sizes of marker proteins are shown at the right-hand sides of the blots and the sizes of the immunoreactive bands appear on the left-hand sides.

Fig. 1A shows that the nuclear sonicate contained a major EE-related polypeptide of 55 kDa, less prominent ones of 130, 66 and 63 kDa and minor polypeptides of 145, 95, 72 and 37 kDa. The results for the serial salt extracts show that the 63 and 55 kDa EE-related polypeptides were the major species bound tightly to the chromatin. Although the photograph shown is truncated, there were no immunoreactive polypeptides of less than 50 kDa in size detected on the original immunoblot. The immunoblot of the nuclear sonicate and NaCl extracts made in the absence of DCIC (Fig. 1B) showed all of the same polypep-
tides that were seen in the presence of DCIC except for the 145 kDa polypeptide. The minor polypeptides present in the nuclear sonicate can also be seen in the salt extracts indicating that they were also extracted in the absence of DCIC from chromatin with increasing salt concentrations. An additional small polypeptide of 32 kDa was prominent in the nuclear sonicate in the absence of DCIC (Fig. 1B). When extracts were made with 0.5 M NaCl, the signal from the 32 kDa species was very prominent and the signals from the 55 to 63 kDa polypeptides only just detectable. These results indicate that the 55 and 63 kDa polypeptides were converted to the 32 kDa polypeptide by limited proteolysis either during sonication or on extraction with 0.5 M salt. A DCIC-sensitive chromatin-bound protease that makes this conversion may thus be activated on perturbing the chromatin structure. It is clear from these results that the human nuclear EE, like the EE of *Neurospora* nuclei (Ramotar et al., 1987), is very sensitive to proteolysis.

The immunoblot of Fig. 1B also shows there are EE-related polypeptides bound very tightly to the nuclear matrix fraction, the major ones in the 55 to 66 kDa range being of the same sizes as those seen in the nuclear sonicate and released from chromatin by salt extraction. An identical pattern of polypeptides was observed for the nuclear matrix derived from cell lysates made in the presence of DCIC (data not shown).

An immunoblot of unfraccionated and fractionated ER-cytosol with the purified antibody raised to *Neurospora* EE is shown in Fig. 2A. The most prominent signal corresponds to a 55 kDa polypeptide associated with the ER membrane fraction which contains the highest amount of total EE. Minor immunoreactive polypeptides of 145, 130, 95, 72, 66, 57, 48, 46, and 37 kDa were also detected in the ER-cytosol fraction (see also Fig. 4C). Many of these immunoreactive peptides are of the same sizes as those detected in nuclei (see above). The major 55 kDa and minor 57 kDa polypeptides were localised in the isolated ER-membrane fraction. The major cytosolic polypeptide detected with this antibody was one of 95 kDa (Fig. 2A). The cytosolic 95 kDa species co-purified about 10-fold with trypsin-activatable EE activity when cytosol was chromatographed on DEAE-Sepharose as described above and after storage in denaturaton buffer at −20°C only a 63 kDa polypeptide was detected.

Much smaller EE-related species were recovered (Fig. 2B) when the CEM ER-cytosol fraction was prepared in the absence of protease inhibitors. The major polypeptide recognised was only 40 kDa, while minor species of 55, 46, 24, 22, 20 and 18 kDa were also detected. This result also indicates an exquisite protease sensitivity of the mammalian EE.

The same major 63, 55 and 95 kDa polypeptides detected with antibody to *Neurospora* EE in ER-cytosol fractions of CEM cells have also been detected in the presence of DCIC in ER-cytosols from MOLT-4 cells (see below) and from several other mammalian sources including human HL-60 cells, preB Namalwa cells, HeLa cells, monkey COS cells and mouse liver. This indicates that the occurrence of EE may be ubiquitous in mammalian cells. A major 67 kDa polypeptide has also been detected on immunobots of crude extracts of Chinese hamster ovary cells and some human ataxia telangiectasia lines with antibody raised to the monkey CV-1 cell EE (Liu et al., 1995). It corresponds to one of the minor species found in the ER-cytosol of CEM cells.

When the EEs of ER-cytosol fractions of CEM cells (control) and preB Namalwa ρ0 cells were assayed in the same experiment, the levels of active EE found were, respectively, 3, 6 and 3 U/10⁸ cells and the levels of total EE were, respectively, 58, 69 and only 5 U/10⁸ cells. An immunoblot revealed major ER-membrane EE species of 63, 57 and 55 kDa in both the CEM and ρ0 cell fractions but only one major 63 kDa ER-membrane polypeptide in the Namalwa ρ0 ER-cytosol fraction. However, on re-blotting duplicate samples after further storage (two weeks) at −20°C, all three fractions contained the 63, 57 and 55 kDa EE species. Assays of the duplicate Namalwa ρ0 and ρ0 ER-fractions yielded, respectively, 10 and 34 U/10⁸ cells of active EE and 66 and 67 U/10⁸ cells of total EE. Thus, the Namalwa ρ0 63 kDa polypeptide had undergone partial limited proteolysis to yield the 57 and 55 kDa species on aging, considerable spontaneous activation, and now showed a total EE equivalent to that in the ρ0 line. These observations showed that the Namalwa ρ0 63 kDa EE was not initially trypsin-activatable.

**Activity gel analysis of EE polypeptides**

Fig. 3A shows the results of activity gel analysis of a freshly prepared CEM nuclear sonicate on ss-DNA gels in the presence of either 5 mM Mg2+ or 5 mM Mg2+ and 2 mM Ca2+. A corresponding immunoblot of this fraction with antibody to *Neurospora* EE, heavily loaded to reveal minor bands, is shown in Fig. 3B. In both the ss-DNA and ds-DNA gels shown in Fig. 3A, it can be seen that there are almost as many bands of activity as there are immunoreactive bands indicated on the left-hand sides of the blots and the positions of the immunoreactive bands indicated on the right-hand sides.
In general, they are of the same sizes as the immunoreactive bands, with prominent active bands of 101, 46, 45, 35, 30 to 34, 28 and 18 to 22 kDa and minor active bands of 66, 60, 40 and 37 kDa. The immunoreactivity of the smallest active species is likely low since it becomes apparent only when a major fraction of the EE has undergone extensive proteolysis (cf Fig. 2B). Significantly, the two largest immunoreactive bands of 145 and 130 kDa did not show any activity. This is consistent with the idea that they are inactive precursor forms of EE. As expected, the smaller polypeptides which renature more efficiently show proportionally more activity. One notable exception to the general pattern was that the prominent immunoreactive band of 95 kDa (Fig. 3B) showed activity with ss-DNA but no activity with ds-DNA (Fig. 3A). In addition, the minor immunoreactive 101 kDa band surprisingly shows as much ss-DNase activity as the more prominent 95 kDa band. Finally, the major 55 kDa immunoreactive band shows only very low ss-DNase activity but significant ds-DNase activity. It is possible that portions of the 95 and 55 kDa polypeptides carry inactivating covalent modifications.

When the two ss-DNA gels shown in Fig. 3A are compared, it is apparent that more activity is seen in the presence of both divalent metal ions, especially for the 101, 95, 40, 35 and 18 to 22 kDa bands. This is consistent with the reported synergistic activation of mammalian EE by Mg\(^{2+}\) and Ca\(^{2+}\) (Couture and Chow, 1992). A control gel treated in the same way but renatured in the presence of 2 mM EDTA instead of divalent metal ions clearly indicated that there was little or no activity expressed in any of these bands except those corresponding to the 45 and 30 to 34 kDa polypeptides (cf Fig. 4A). These species thus represent either metal ion independent activities or they are DNA-binding proteins which block the access of the fluorescent dye to the DNA. Their properties have not been further investigated here.

Fig. 4A and B show activity analyses of freshly prepared CEM ER-cytosol fraction using ss-DNA and ds-DNA gels activated in the presence of EDTA alone or with Mg\(^{2+}\) and/or Ca\(^{2+}\) as indicated. The ER-cytosol fraction was extracted in the presence of the full complement of protease inhibitors including DCIC. A corresponding immunoblot for this fraction, heavily loaded to allow detection of the minor EE-related species, is shown in Fig. 4C. The patterns of active

![Fig. 3](image_url)

**Fig. 3.** (A) Activity gels for a CEM cell nuclear sonicate (200 μg protein per lane) run on either ss-DNA gels and activated as indicated with 5 mM Mg\(^{2+}\) or 5 mM Mg\(^{2+}\) and 2 mM Ca\(^{2+}\) or run on a ds-DNA gel and activated with both metal ions. Marker proteins were run in the left-hand lane. (B) The corresponding immunoblot for 500 μg nuclear sonicate protein. The sizes of marker proteins are seen on the left-hand side of the gels and the active (A) or immunoreactive bands (B) on the right in each panel. The sizes indicated for the activity bands in A were for the ss-DNA gel (centre two lanes).

![Fig. 4](image_url)

**Fig. 4.** Activity gels for the CEM cell ER-cytosol fraction. (A) ER-cytosol (200 μg protein) run on ss-DNA gels and activated as indicated in the presence of either 2 mM EDTA, 5 mM Mg\(^{2+}\) and 2 mM Ca\(^{2+}\), 5 mM Mg\(^{2+}\) alone or 2 mM Ca\(^{2+}\) alone. An empty lane is shown on the left. The third and right-hand lanes contain marker proteins. (B) ER-cytosol (500 μg protein) run on a ds-DNA gel and activated in the presence of 5 mM Mg\(^{2+}\) and 2 mM Ca\(^{2+}\). (C) Corresponding immunoblot for 500 μg ER-cytosol protein probed with antibody raised to purified Neurospora EE. The sizes of the marker proteins are indicated at the right and the sizes of the active and immunoreactive bands are indicated at the left on each panel.
polypeptides detected on ss-DNA gels is remarkably similar to that detected for sonicates of the CEM nuclei (Fig. 3A) showing multiple active species that correspond in the main to immunoreactive species. Again, the 95 kDa species showed ss-DNase, but no ds-DNase activity. In addition, the major immunoreactive 55 kDa polypeptide showed little or no DNase activity. Aged ER-cytosol fractions have not been subjected to activity gel analysis, but an active 55 kDa species has been detected by this method in stored preparations of isolated ER-membranes (data not shown). While the activities of most of the polypeptides seen in Fig. 4A were strongly suppressed in the presence of EDTA, those of the 45 kDa species and the broad band centering at 31 kDa remained high as seen in nuclear sonicates (see above). These bands are possibly metal ion-independent nucleases or DNA-binding proteins.

As for the nuclear EE, ER-cytosol EEs of 101, 95, 46, 35, 32 and 18 to 22 kDa can be seen in Fig. 4A to exhibit some activity in the presence of either Mg2+ or Ca2+ but show greater activity in the presence of both metal ions. Also the immunoreactive 145 and 130 kDa bands seen in Fig. 4C do not show any activity on either of the ss-DNA or ds-DNA gels. They are likely EE precursor forms. Finally, a broad band of activity at 57 to 63 kDa is seen with ds-DNA, but this had only very low activity with ss-DNA. This corresponds to immunoreactive ER-membrane bands of 57 to 63 kDa often appearing above the inactive major 55 kDa immunoreactive EE-related polypeptide.

**Chromatin DNA laddering in an isolated nuclei/cytosol system**

CEM nuclei from 1.5×10⁸ cells were incubated with cytosol from the same number of untreated CEM cells. Cytosols were prepared in lysis buffer containing 5 mM Mg²⁺ and 0.1% NP-40. A parallel incubation was performed in the presence of 50 μM TLCK, which has been reported to block laddering of chromatin DNA during apoptosis of intact thymocytes (Fearnhead et al., 1995) and the formation of 50 kbp fragments and DNA ladders in isolated rat liver nuclei (Zhivotovsky et al., 1994b). Although there was some slight degradation of the DNA within the first hour as seen from the entry of all of the DNA into the agarose gel, extensive degradation of the DNA to form ladders was not observed until between 12 and 24 hours (Fig. 5A). In the presence of TLCK, the DNA laddering was clearly reduced but not abolished. DNA laddering was also observed when CEM nuclei were incubated under the same conditions in the absence of cytosol, but not in the presence of 6 mM EDTA.

Fig. 5B shows many changes in the EE-related polypeptides during the incubation of the nuclei/cytosol. The largest EE-related polypeptides either disappeared or were depleted at early times, but at slower rates in the presence of TLCK. Changes in several of the immunoreactive polypeptides correlated with the appearance of DNA ladders between 12 and 24 hours. These include the substantial increases in the levels of
polypeptides of about 66, 63, 40 kDa and the appearance of new EE-related polypeptides of 46 and 18 to 22 kDa. Although there was slight over-exposure in development of the blot, the levels of the 18 to 22 kDa polypeptides were clearly reduced in the presence of TLCK.

Changes in EE-related nuclear polypeptides during apoptosis

CEM and MOLT-4 cells were pre-treated in culture with doses of VP-16 and PODO that were expected to induce apoptosis (see below). The two different leukaemic cell lines were chosen for study because they have different sensitivities to these drugs, while the two drugs, although they both induce apoptosis in these cell lines, have different modes of action. VP-16 targets nuclear topoisomerase II and PODO targets cytosolic tubulin.

Flow cytometric DNA histograms for untreated control CEM cells and drug-treated CEM cells are shown in Fig. 6A and the corresponding agarose gels are shown in Fig. 6B. In the first experiment, CEM cells were either untreated or pre-treated with 10 μM VP-16 for 8 hours or 1 μM PODO for 15 hours. There was no apoptosis in the control cells assessed by either technique. The DNA histograms (Fig. 6A) indicate that the VP-16 treatment in this case caused an S-phase block without showing an appreciable less than 2N DNA peak, while the corresponding gel (Fig. 6B) showed that no DNA laddering had occurred. Apoptosis was seen in the PODO-treated CEM cells by both techniques. In a second experiment with CEM cells the dose of VP-16 was doubled, to 20 μM for 8 hours. In this case, apoptosis was observed by both techniques in the drug-treated CEM cells.

Fig. 7 shows the corresponding immunoblots for the two experiments. Here the nuclear sonicates of untreated CEM cells and of CEM cells pre-treated either with 10 or 20 μM VP-16 for 8 hours or with 1 μM PODO for 15 hours were probed with antibody raised to Neurospora EE (Fig. 7A and B). The drug treatments of the CEM cells did not result in any appreciable cell necrosis as judged from the very low numbers of trypan blue positive cells, although the PODO treatment in particular reduced the cell number significantly (Fig. 7A). Both drug treatments resulted in significant increases of total EE activity detected per 10^8 cells in the nuclear sonicates after trypsin activation, respectively 140% and 190% over the control for the VP-16 and PODO treatments in the first experiment (Fig. 7A) and 315% over the control for the VP-16 treatment in the second experiment (Fig. 7B). This suggests that the drug treatments either caused a dose-dependent uptake of EE into the nuclei or unmasking of endogenous trypsin-activatable nuclear EE or stimulated both processes.

The immunoblots for untreated and VP-16-treated CEM cells which arrested in S-phase and yielded no evidence of DNA laddering (Fig. 6, exp. 1), when probed with antibody to Neurospora EE, showed only two slight differences from that for control cells in the spectrum of EE-related polypeptides (Fig. 7A). Appearance of a new minor polypeptide of 72 kDa in the nuclear sonicate of VP-16-treated cells and an increased level of the 63 kDa species were noted. A major polypeptide of 55 kDa and minor polypeptides of 145, 130, 95, 60, and 46 kDa were common to both nuclear sonicates. On the other hand, in CEM cells treated with twice the dose of VP-16 (Fig. 7B) which showed evidence of apoptosis (see Fig. 6), there was an increase in the signal from the 95 kDa polypeptide, reduction in the signal from the 72 kDa polypeptide relative to the control and marked increases in signals from 43 and 37 kDa polypeptides. The nuclear sonicate of the PODO-treated cells in which apoptosis was detected (see Fig. 6), showed a new small EE-related polypeptide of 37 kDa and evidence of breakdown of larger polypeptides, in particular those of 145, 130 and 46 kDa (Fig. 7A). Thus, the appearance of small EE-related polypeptides correlated with the appearance of extensive chromatin DNA fragmentation. This indicates that the small EE-related species (below 50 kDa) may be responsible for the DNA laddering observed.

Pre-treatments of MOLT-4 cells in culture with either 50 μM VP-16 for 8 hours or 25 μM PODO for 15 hours also resulted in appearances of small EE-related polypeptides which corre-

Fig. 6. (A) Flow cytometric DNA histograms for untreated (UNTR) CEM cells and CEM cells treated with either 10 μM (experiment 1) or 20 μM (experiment 2) etoposide (VP-16) for 8 hours or treated with 1 μM podophyllotoxin (PODO) for 15 hours (experiment 1). The peaks indicating less than 2N DNA content are at the left of the arrows. (B) Agarose gel electrophoresis of DNA extracted from the untreated or drug-treated cells in the two experiments.
lated with the appearance of DNA ladders. Only the corresponding immunoblot is shown here (Fig. 7C). In particular, the signal from the 32 kDa species was just detectable in the nuclear sonicate from control cells, but was greatly enhanced by both drug treatments. In addition, a 22 kDa species appeared in response to both drug treatments but was absent in the nuclear sonicate of untreated MOLT-4 cells. In this experiment, a 66 kDa polypeptide also appeared in the nuclear sonicates of both drug-treated MOLT-4 cells. The signal from the 72 kDa species, just detectable in the control and VP-16-treated cells, was noticeably enhanced in the nuclear sonicate of PODO-treated cells. Thus, in both cell lines pre-treatments with either VP-16 or PODO which induce apoptosis result in very similar changes in EE-related polypeptides which implicate the smaller EE species in the extensive chromatin DNA fragmentation which occurs during apoptosis. However, unlike the result for CEM cells, the drug treatments did not cause increases in total EE levels in the nuclei. In fact, slight losses of 22 to 25% were observed (Fig. 7C).

Changes in EE-related ER-cytosol polypeptides during apoptosis

CEM and MOLT-4 cells treated with an equal dose of PODO

CEM and MOLT-4 cells were treated with 10 μM PODO over a 24 hour time period, sampling treated and DMSO (drug solvent) control cells every 6 hours. It can be seen in Fig. 8A and B, that there was a marked decrease in the total EE in the ER-cytosol fraction of the CEM cells relative to the controls starting at 12 hours of treatment, but only a small decrease in the total EE was observed in the ER-cytosol fraction of the MOLT-4 cells relative to the controls. The appearance of DNA ladders was first detected in the CEM cells at 12 hours and increased at both 18 and 24 hours (Fig. 8C). In this experiment no laddering was detected in the DNA from treated MOLT-4 cells at any of these time points and no DNA fragmentation occurred in the control CEM and MOLT-4 cells (Fig. 8C).

Cytological examinations (Fig. 8D) revealed apoptotic bodies and many disordered mitoses in the PODO-treated cells. Analysis showed, respectively, 9 and 195 apoptotic bodies or clusters of apoptotic bodies per 300 control and PODO-treated CEM cells and, respectively, 16 and 115 apoptotic bodies per 300 control and PODO-treated MOLT-4 cells at 24 hours. These results show that there was about one-half as much apoptosis in the MOLT-4 cells as in the CEM cells by morphological criteria even though no DNA laddering was detected (see Discussion).

Immunoblots for the ER-cytosol fractions of control and 10 μM PODO-treated cells at all time points over-exposed to reveal detail are seen in Fig. 9A and B. In the PODO-treated CEM cells (Fig. 9A), the levels of the 130, 95, 72 and 37 kDa EE-related polypeptides increased in the first 6 hours relative to the DMSO control and thereafter decreased at various times following the first detection of DNA laddering at 12 hours. The pattern of changes in the levels of these polypeptides is consistent with a model in which the 130 kDa polypeptide is a
precursor of the 95, 72 and 37 kDa polypeptides which undergo progressive proteolytic breakdown during chromatin DNA fragmentation in the drug-treated cells. The signals from the major CEM ER-membrane 55 and 63 kDa polypeptides changed independently of those from other EE-related polypeptides. The signals from these two polypeptides were about 1:1 in ratio at each time of treatment (Fig. 9A) and slowly increased relative to the DMSO controls during the 12 to 24 hour period of DNA degradation.

The interpretation of the above data for PODO-treated CEM cells suggesting that proteolysis of the EE-related 130, 95, 72 and 37 kDa polypeptides is correlated with the extensive DNA laddering is reinforced by the results obtained with PODO-treated MOLT-4 cells in which no DNA laddering was detected (Fig. 9C). Here the immunoblot of the ER-cytosol fractions shows signals from the 130, 95 and 37 kDa polypeptides appearing at 6 hours in both the DMSO control and PODO-treated cells as for CEM cells, but all species persist for the full 24 hour period of the drug treatment. The signals from the ER-membrane 63 and 55 kDa species also increased slowly in the PODO-treated MOLT-4 cells as for treated CEM cells.

The results of the experiment described in Figs 8 and 9 indicated that CEM and MOLT-4 cells had different sensitivities to PODO for the induction of apoptosis, the MOLT-4 cells being much more resistant. Preliminary results for VP-16 induction of apoptosis in the two cell lines yielded similar results. Therefore, systematic studies of the dose responses of these two cell lines to VP-16 and PODO were made (data not shown) in order to determine appropriate doses which would induce apoptosis, respectively, in 12 and 24 hours without causing appreciable secondary necrosis. These studies confirmed that MOLT-4 cells were about 5 times more resistant to VP-16 and about 10 times more resistant to PODO than CEM cells. They indicated that doses of VP-16 in the range of 10 to 20 μM for CEM cells and 25 to 50 μM for MOLT-4 cells over 12 hours and doses of PODO of about 1
μM for CEM cells and about 25 μM for MOLT-4 cells over 24 hours would be suitable for the time course experiments shown below.

Time courses for CEM and MOLT-4 cells treated with VP-16

The results of a time course of treatment of CEM cells with 10 μM VP-16 for a 12 hour period are shown in Fig. 10A and B, and Fig. 11. The flow cytometric DNA histograms presented in Fig. 10A show that a less than 2N DNA peak began to appear at 4 hours for the VP-16-treated cells. Agarose gel electrophoresis of the extracted DNA (Fig. 10B) showed that the DNA laddering also began to be apparent at 4 hours. No DNA laddering was detected in the control cells.

The immunoblot for the EE-related polypeptides at these times of VP-16 treatment is shown in Fig. 11. In this experiment, there was no appreciable secondary necrosis of the CEM cells over the 12 hour treatment period as seen by the trypan blue exclusion test. It can be seen that at 4 hours of treatment onward, there were marked decreases in the signal from the ER membrane-associated 55 kDa polypeptide. The total ss-DNase activity was 54 U/10^8 cells in the control cells and decreased to 34 U/10^8 cells by 12 hours. Increases in the cytosolic 95 kDa species were seen after 4 hours. It is not clear whether the increases in the 95 kDa species result from new synthesis of EE in response to the drug or, more likely as the time of exposure to VP-16 increases, is due to a gradual leakage from the nuclei.

MOLT-4 cells treated with 50 μM VP-16 were also followed over a 12 hour time course (data not shown). Detectable apoptosis also started at 4 hours and by 12 hours most of the population was found in the less than 4N DNA peak, while a maximum of only 5% of the cells became trypan blue positive. Similar changes were observed to occur in the ER-membrane 55 kDa and cytosolic 95 kDa EE polypeptides following the detection of DNA laddering at 4 hours as seen in CEM cells. The levels of trypsin-activatable EE averaged 109 U/10^8 cells in the controls and decreased to 53 U/10^8 cells at 12 hours in the VP-16-treated MOLT-4 cells.
Time courses for CEM and MOLT-4 cells treated with PODO

When CEM cells were treated with 1 μM PODO and sampled over a 24 hour period, apoptosis was first detected by flow cytometry (data not shown) and agarose gel electrophoresis of extracted DNA at 9 hours (data shown in Fig. 12B). By 12 hours nearly all of the DNA was in laddered form.

The immunoblot of the ER-cytosol fractions of the 1 μM PODO-treated CEM cells (Fig. 13) shows that the signals from the ER membrane-associated 55 and 57 kDa species increased markedly over the DMSO control in the first 3 hours and then steadily decreased over the next 9 hours as the degradation of DNA proceeded. The increases in signals from the 95 kDa species at 15 and 24 hours with respect to the DMSO control and the appearance at 24 hours of signals from smaller immunoreactive polypeptides of about 46, 37 and 32 kDa indicate that EE polypeptides were being released from deteriorating nuclei. By 24 hours, there were 14% trypan blue positive cells which indicated that appreciable secondary necrosis was beginning to occur. The levels of trypsin-activatable EE decreased by much smaller amounts in this experiment than in CEM cells treated with 10 μM PODO (cf Fig. 8A). Fig. 13 shows that the levels averaged 68 U/10^8 cells in the controls but increased to 87 U/10^8 cells at 3 hours and then decreased to 73 U/10^8 cells as the DNA degradation proceeded and the ER-membrane EE polypeptides were partially depleted.

MOLT-4 cells were treated with 25 μM PODO and also monitored over a 24 hour period. As with the CEM cells, tetraploid MOLT-4 cells with less than 4N DNA were first detected at 9 hours by flow cytometry (Fig. 12A) and DNA laddering began to be apparent at this time as well (Fig. 12B).

Most of the DNA was in ladder form by 12 hours but there was still little loss in cell number and little increase in trypan blue positive cells at this time (Fig. 14). By 24 hours, the cell number had dropped appreciably and the trypan blue positive cells had increased from only 2.5% at 15 hours to 67% (Fig. 14) indicating that substantial secondary necrosis was occurring between 15 and 24 hours.

The immunoblot for the ER-cytosol fractions from the PODO-treated MOLT-4 cells is shown in Fig. 14. When the double loading of the 12 hour sample is taken into account, the pattern of EE polypeptide changes follows closely that seen in the previous experiment for PODO-treated CEM cells. The signal from the ER-membrane 55 kDa EE polypeptide was depleted from the maximum at 6 hours over the period in which
DNA degradation occurred to a minimum at 15 hours without detectable change in the cytosolic 95 kDa EE species. This was accompanied by a modest loss of total EE from 106 U/10^8 cells to 72 U/10^8 cells at 12 hours.

**DISCUSSION**

**Endo-exonuclease is a major nuclease in human leukaemic cells**

This work shows for the first time that over 95% of the nuclease acting optimally at neutral pH to degrade ss-DNA in human leukaemic CEM and MOLT-4 cell nuclei is a Mg\(^{2+}\)-dependent, Ca\(^{2+}\)-stimulated endo-exonuclease similar to the major activity present in *Neurospora* nuclei (Ramotar et al., 1987) and the EE in monkey CV-1 cells (Couture and Chow, 1992). Although this activity has not been purified from human leukaemic cells, like the *Neurospora* EE, it is very sensitive to inhibition by an excess of EDTA over divalent metal ion, by 25 \(\mu\)g/ml ATA and by 2 mM Zn\(^{2+}\) and it cross-reacts with antibody raised to the purified *Neurospora* EE. CEM cell EE is present in serial salt extracts of nuclei (Fig. 1) almost entirely in inactive, but trypsin-activatable, forms similar to those found also in *Neurospora* (Fraser and Cohen, 1983; Hatahet and Fraser, 1989). A key identifying feature of the fungal, monkey CV-1 and human leukaemic cell nuclear EEs is the 2:1 ratio of the ss-DNase and poly rAase activities, respectively, the latter seen by simultaneous activation with trypsin (see Introduction and Results).

The sizes of a number of the immunoreactive human nuclear polypeptides (Figs 1 and 3B) are very similar to immunoreactive and active polypeptides detected in nuclei of *Neurospora* (Ramotar et al., 1987), in particular those of 95, 72, 66, and 37 kDa. When analysed on activity gels (Fig. 3), except for the polypeptides of 145 and 130 kDa, the immunoreactive human polypeptides had Mg\(^{2+}\)-dependent and Ca\(^{2+}\)-stimulated ss- and ds-DNase activities like the purified CV-1 EE (Couture and Chow, 1992). Two notable exceptions were the 95 kDa polypeptide which showed activity with ss-DNA but not with ds-DNA and the major nuclear 55 to 66 kDa immunoreactive bands tightly bound to the chromatin and nuclear matrix of CEM cells (Fig. 1) which had no detectable ss-DNase activity and just detectable ds-DNase activity (Fig. 3). It is possible that these EEs are inhibited (see below) by covalent modification such as phosphorylation or by ADP-ribosylation as described for a bovine Ca\(^{2+}\), Mg\(^{2+}\)-endonuclease with both DNase and RNase activities (Hashida et al., 1982; Tanaka et al., 1984). Baxter et al. (1989) observed by activity gel analysis a doublet of about 55 kDa in nuclear extracts of untreated CEM-C7 cells undergoing apoptosis but did not detect these active polypeptides in nuclear extracts of untreated CEM-C7 cells. This indicates that the 55 kDa species, or its 63 kDa precursor, is activated during apoptosis. More recently, a triplet of active 42, 45 and 50 kDa nucleases was found to appear in nuclei of human Jurkat cells induced to undergo apoptosis by different agents (Zhang et al., 1995). In this case, it was shown that none of the triplet nucleases cross-reacted with antibody raised to pancreatic DNase I.

Active polypeptides have been detected in mammalian nuclei by other investigators. The 101 kDa active band observed in this work may correspond to an active polypeptide of about 97 kDa detected by activity analysis on ds-DNA gels in nuclear wash fractions (nucleoplasm) of rat 5125 tc hepatoma and PC12 pheochromocytoma and of human MCF-7 breast and DU-145 prostatic carcinoma cells (Pandey et al., 1994). When these nucleoplasm were added to isolated nuclei from the same cell lines, laddering of the chromatin DNA was observed. Four polypeptides of 120, 54, 31 and 28 kDa having...
nicked activity with plasmid DNA (endonuclease activity) have been detected in extracts of isolated rat liver nuclei in the presence of PMSF (Lebedeva et al., 1995). In the absence of PMSF additional active species were found showing that the rat nuclease was also sensitive to proteolysis. Rat liver nuclei contain a nuclease with both endo- and exonucleolytic activities (Hibino et al., 1991) which could be a form of EE. Mouse liver was found here to contain EE-related polypeptides (see Results).

As in Neurospora (Fraser and Cohen, 1983), a second large pool of EE, mainly in trypsin-activatable form, has been identified in the ER-cytosols of CEM and MOLT-4 cells (Table 1). It is present at twice the level found in CEM nuclei and four times the level found in MOLT-4 nuclei. A minor amount of this activity (15%) was found in the cytosol associated with a 95 kDa EE with the same properties as its nuclear counterpart (see above). This soluble inactive EE was immunoprecipitatable with antibodies raised to both the Neurospora and monkey CV-1 cell endonucleases (Table 2).

Most of the ER-cytosol EE was inactive, but trypsin-activatable EE bound to the ER membranes and with properties identical to those of the nuclear EE (Figs 2 and 4). It also showed trypsin activation of ss-DNase and poly rAase activities in the characteristic ratio of 2:1. DNase I-like activity implicated in apoptosis has been reported to be localised to the ER-membranes of thymocytes (Petisch et al., 1993), but since no trypsin activation of this activity was reported and since RNase activity was not determined, the possible identity of this DNase I-like activity with EE is not yet known. It has been pointed out that the sugar non-specific nucleases like endoexonuclease have been confused in the past with DNase I-like enzymes (Sierakowska and Shugar, 1977).

The ER-cytosol fraction, like nuclei, also contained major immunoreactive 55 to 63 species (Figs 2 and 4C) which show only little DNase activity (Fig. 4). As suggested for its nuclear counterpart, much of this EE may also be inhibited by covalent modification since an active 55 kDa polypeptide was detected in aged ER membrane preparations. Further evidence for blocked trypsin-activatable EE was found for the ER-cytosol fraction derived from Namalwa cells (see Results). Active EE was recovered after limited proteolysis of the 63 kDa EE-related polypeptide during aging and a 55 kDa polypeptide became prominent. It is relevant here that a ss-specific endonuclease of 54 kDa acting on both DNA and RNA was purified previously from the ER of rat liver (Koudou et al., 1981, 1987). This activity is very likely derived from EE just as the ss-endonuclease was found to be derived from endo-exonuclease in Neurospora (Chow and Fraser, 1983; Fraser et al., 1986). The mechanism of masking of the human leukaemic cell ER membrane species in fresh preparations is not yet known.

**Different inactive forms of endo-exonuclease**

At least three types of nuclear and ER-cytosol inactive EE forms have been identified (compare Figs 1 and 2 with Figs 3 and 4). The first type includes the immunoreactive polypeptides of 145 and 130 kDa which show no activity in activity gel analysis. These are assumed to be precursor forms of EE. The second type of inactive EE reveals active polypeptides of 101 to 18 kDa on activity gel analysis (Figs 3 and 4) which correspond to EE-inhibitor complexes. A third type of inactive EE is immunoreactive but it is not initially activated with trypsin and it is not revealed initially by activity gel analysis. However, it becomes trypsin-activatable on aging. Inactive EE was more accessible to activation by trypsin after pre-incubation with divalent metal ions but additional trypsin-activatable EE may be masked (see above). The masking mechanisms are not yet known. All of these types of inactive EE have been seen previously in Neurospora (Hatahet and Fraser, 1989; Ramotar and Fraser, 1989). In Neurospora, a small highly specific heat-stable and heat shock inducible inhibitor of EE was isolated. In Neurospora, the detection of several larger inhibitory polypeptides of sizes identical to the larger EE forms led to the suggestion that the inhibitor may have been derived, like EE itself, via limited proteolysis from a large inactive polypeptide precursor, possibly a common precursor. Attempts to find a similar heat-stable EE inhibitor in human leukaemic cell extracts have so far failed.

The presence of inactive EE forms in human leukaemic cells would serve to protect the DNA and RNA in the nucleus and the RNA in the ER-cytosol from unwanted degradation during normal cell growth. Their presence indicates that EE is under tight regulation. When apoptosis is triggered, fragmentation of 28 S rRNA of both free and bound ribosomes in the extranuclear compartment is coordinated with chromatin DNA fragmentation in the nucleus (Houge et al., 1993, 1995). If EE plays a role in the apoptotic process as proposed here, it is expected that the regulation of EE activation on the ER-membranes and in the nucleus will be coordinated and complex since the apoptotic process is highly ordered. A major facet of this regulation may be proteolysis.

**Proteolysis of EE polypeptides in response to apoptotic stimuli**

Limited proteolysis of chromatin bound EE to yield a 32 kDa species was also activated in low salt washed chromatin by extraction with 0.5 M NaCl in the absence of DCIC (Fig. 1B). It is not yet known whether the 32 kDa EE can specifically make internucleosomal cleavages in chromatin DNA. A salt activated protease is one of three distinct proteases described in yeast nuclei (Motizuki et al., 1988). In nuclei isolated from untreated CEM cells which showed spontaneous chromatin DNA fragmentation (Fig. 5), and in nuclei isolated from CEM and MOLT-4 cells undergoing apoptosis (Fig. 7), the appearance of new small EE-related polypeptides correlated with the appearance of the DNA ladder. These results indicate that small EE species are specifically responsible for the DNA cleavage observed. The sizes of some of these new polypeptides are remarkably similar or identical to those of different endonucleases implicated by others in apoptosis of lymphoid cells and thymocytes: 18 kDa (Caron-Leslie et al., 1991); 40 kDa (Ucker et al., 1992); 22 kDa (Nikonova et al., 1993) and 32 kDa (Shikawa et al., 1994). Interestingly, a higher molecular mass form of NUC18 was not able to induce laddering of chromatin DNA (Caron-Leslie et al., 1991). Of all of these candidate apoptosis nucleases, only NUC18 has been tested and shown to have RNase activity. The simplest hypothesis to account for the multiple endonucleases that have been implicated in apoptosis to date is that they are different forms of EE. An alternative hypothesis is that a variety of different endonucleases are involved. This now seems less likely in view of the present results.

The uptake of total EE into the CEM nuclei in response to
both VP-16 and PODO (Fig. 7a and B) and lack of uptake in response to these drugs into MOLT-4 nuclei (Fig. 7C) also suggests a role for EE in drug-induced apoptosis. The more drug-resistant MOLT-4 line is partially deficient in nuclear EE and untreated cells have a greater store of EE in their extranuclear compartment (Table 1) consistent with a partial defect in uptake of EE into their nuclei. MOLT-4 cells may be more drug-resistant because they cannot call into play additional EE from the ER-cytosol during apoptosis and must rely on activation of the deficient nuclear EE for chromatin DNA degradation. It should be noted that the resistance of MOLT-4 appears greater when estimated by the criteria of the appearance of cells with less than 4N DNA seen by flow cytometry and DNA laddering, but when compared at the same dose of PODO by morphological criteria (Fig. 8D) the resistance to PODO was only about two times that of the CEM cells. Presumably, the DNA fragmentation in the MOLT-4 cells has been sufficient to collapse the chromatin structure and result in large DNA fragments, but not sufficient to generate DNA ladders. Limited fragmentation of chromatin DNA is sufficient to induce apoptosis (Oberhammer et al., 1993; Bicknell et al., 1994; Cohen et al., 1994).

Finally, the timing of onset of limited proteolysis of EE-related polypeptides in the ER-cytosols of both CEM and MOLT-4 cell treated with VP-16 or PODO correlates well with the timing of onset of DNA laddering. VP-16 was seen to trigger EE proteolysis and DNA laddering starting at about four hours in both cell lines (Figs 10, 11 and Results). PODO, on the other hand, triggered both processes starting at about nine hours in both cell lines (Figs 12-14). The differences in timing likely reflect differences in the signalling pathways for the two drugs to activate nuclear EE for chromatin DNA fragmentation.

Significant losses of the large ER membrane-associated polypeptides were the main responses to both drugs, while the cytosolic 95 kDa EE remained stable or increased (Figs 11, 13 and 14). In most cases, the membrane EE losses occurred without the concomitant appearance of the small EE polypeptides and were accompanied by only partial losses of total EE activity. Increased levels of the cytosolic 95 kDa species would account for at least part of the remaining total EE and more may be present in EE-inhibitor complexes which contain small EE polypeptides with poor immunoreactivity (cf Fig. 2B). In the case of CEM cells, portions of ER-membrane EE may have been mobilised and entered the nuclei. However, the losses in MOLT-4 cells, which showed no uptake of EE into nuclei in response to the drugs, would be explained by proteolysis in the cytosol. At the latest times of drug treatment, when small EE-related polypeptides did appear in the ER-cytosols of drug-treated cells (Fig. 13), the cells were undergoing appreciable secondary necrosis. Thus, these small EE species may have originated from the nuclei as a result of the leakage from the nuclei.

**Possible roles for the two pools of extranuclear EE**

This work establishes that there are two distinct extranuclear pools of EE in human leukaemic CEM cells (Fig. 2 and Results), a mobile pool present in the cytosol and a second pool immobilised on the ER-membrane fraction. The mobile EE is associated mainly with the 95 kDa EE while the bound EE is associated mainly with the 55-66 kDa species which have very different properties (see above). Since both pools have nuclear counterparts it is reasonable to propose that the extranuclear EEs are precursors of the nuclear EEs. The major ER-membrane species could serve as the origin of the major chromatin bound 63 kDa EE species if there were a mechanism for mobilisation from the ER membranes.

As a framework for further studies on the biological roles of EE, the following model is proposed in which it is assumed that the two pools of cytosolic and ER-membrane bound EEs have basically distinct roles. The mobile cytosolic pool of EE is assumed to be involved normally in DNA repair and possibly in other house-keeping functions involving nicking of the supercoiled chromatin DNA. The implication of EEs in DNA repair, especially combinatorial ds-break repair, has been discussed (see Introduction, and Fraser, 1994). The immobile ER-membrane bound EE can be thought of as a storage reserve form of EE used specifically for apoptosis. The major nuclease activity associated with this form of EE is Mg$^{2+}$, Ca$^{2+}$-dependent ds-DNase when fully unmasked. Its activity may be partially blocked by covalent modification (see Results) as well as being associated with a trypsin-sensitive inhibitor. The binding and release to the cytosol of this ER membrane EE could well be under the control of the bcl-2/bax oncogene family (McConkey et al., 1995), but proteolysis is also likely to play a role in its mobilisation. Activation of this EE in the cytosol compartment could play a role in 28 S rRNA cleavage (Hougue et al., 1993, 1995) and the overall degradation of RNA observed in apoptosis (Darzynkiewicz et al., 1992), while activation of the chromatin/matrix EE would lead to degradation of nuclear DNA and RNA.

Multiple proteases have been implicated in apoptosis (Weaver et al., 1993; Chow et al., 1995; Fearnhead et al., 1995; Voekel-Johnson et al., 1995). A family of ICE-like proteases is now known to be specifically activated during apoptosis (Martin and Green, 1995). One of these, apopain (the CPP32 protease) has high homology to the ced-3 gene product which has been shown to be essential for apoptosis in C. elegans (LaZebnik et al., 1994; Nicholson et al., 1995). Apopain has been found to cleave another nuclear enzyme implicated in DNA repair and apoptosis, namely poly(ADP-ribose) polymerase. It is thus possible that specific activation by apopain and mobilisation of the ER-membrane and nuclear EE would also accompany, if not trigger, apoptosis.

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Endo-exonuclease in apoptosis


