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Biochemical and Biophysical Research Communications 316 (2004) 93–99
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Caspase 3 activation is controlled by a sequence located in the N-terminus of its large subunit☆

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Received 2 February 2004

Abstract

We report that the induction and completion of the apoptotic program is delayed in a doxorubicin-resistant cell line (HL60/ADR). This hindrance to cell death occurred downstream of the multidrug-resistant protein (mrp), a transmembrane transporter. In vitro studies showed that these cells were incapable of correctly activating procaspase 3 (pC3), the main executioner of apoptosis. Sequencing of HL60/ADR pC3 revealed point mutations in a sequence located in the N-terminal region of the large subunit of caspase 3 (C3, amino acids 31–37; i.e., immediately after the propeptide). We called this particular form of C3, the C3 N-terminal modified (C3-NTM), and show that it is partially active when transfected into MCF-7 cells shown to have little or no endogenous pC3. As a deletion of the amino acids 31–37 in wild-type C3 leads to the same phenotype, we conclude that this sequence is involved in C3 activation during apoptosis.

Keywords: Apoptosis; Multidrug resistance; Caspase 3

Apoptosis is a tightly controlled process, which is thought to be involved in many aspects of normal and pathological cell physiology [1,2]. Numerous works have shown that specific proteases are involved in the final phase of apoptosis, which leads to the destruction of the cell. A family of cysteine proteases that specifically cleave after an aspartic acid residue, called caspases, appear to be the principal effectors in the demolition phase of apoptosis [3–8]. Caspases fall into three categories, which are either involved in the regulation of the inflammatory process (inflammatory caspases: e.g., C1, C4, C5, and C13), in the amplification of the apoptotic signal (initiator caspases: e.g., C8, C9, and C10) or in the demolition phase of apoptosis (executor caspases: mainly C3, C7, and C6). The initiator caspases are activated through the interaction of their long prodomains with specialized recruiter/adaptor molecules that are linked to different apoptogenic signals such as cytochrome *c*/apaf-1 in the mitochondrial pathway or FADD/TRADD in the Fas/TNF pathway [3–8]. C8 and C9 transactivate the executor caspases through the cleavage of the procaspase into two subunits, which is followed by the autocatalytic removal of the prodomain [3–8]. This pattern of transactivation has been particularly well documented in the case of C3, which is first cleaved by initiator caspases at an IETD site located between the large and small subunits to produce two subunits of p12 and p20. Next, the prodomain is cleaved from the p20 subunit at the canonic site ESMD to produce the mature p17 subunit [3–8]. The importance of caspases in this self-activating process is emphasized by the fact that endogenous inhibitors of caspases, such as AIPs or dominant negative isoforms of caspases, are powerful inhibitors of apoptosis [6–9].

☆ *Abbreviations:* Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; Ac-LEHD-AMC, acetyl-Leu-Glu-His-Asp-7-amido-4-methylcoumarin; Ac-IETD-AMC, acetyl-Ile-Glu-Thre-Asp-7-amido-4-methylcoumarin; C3, caspase 3; C3-NTM, caspase 3-N-terminal modified; MDR, multidrug resistance; mrp, multidrug-resistant protein; P-gp, P-glycoprotein; pC3, procaspase 3; pC3-NTM, procaspase 3-N-terminal modified; STS, staurosporine.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.02.021

Recent studies have provided evidence that the expression of a multidrug resistance (MDR) profile could be accompanied by a resistance to drugs beyond the MDR substrate range [10]. This "pleiotropic" resistance has been recently linked, in the case of P-glycoprotein (P-gp) expressing cells, to a modification of their sensitivity to apoptosis especially through the inhibition of caspase activation pathways [11–13]. In addition to P-gp, several other related drug transporters (e.g., multidrug-resistant protein (mrp), lung-resistant protein, etc.) are responsible for the MDR phenotype but very little information is available on their relationship with apoptosis. We have thus studied the resistance to apoptosis in the mrp expressing HL60/ADR cell line [14]. We found that these cells express a variant of C3, which is apparently poorly activated during apoptosis because of a series of point mutations in the N-terminal of the p17 subunit.

Materials and methods

Reagents

Unless otherwise specified, all reagents used in this study were from Sigma (St. Quentin, Fallavier, France). Commercial antibodies against C3 and C9 used were from (BD Biosciences, Le Pont de Clair, France). Antibody against actin was from Chemicon (Temecula, CA) and XIAP was from R&D Systems (Abington, UK). The fluorogenic C3, C8, and C9 substrates (Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC) were obtained from Bachem (Bubendorf, Switzerland). Active C3 and C8 were obtained from Oncogene (Cambridge, MA). All cell culture material was obtained from Invitrogen (Cergy Pontoise, France).

Methods

Cell cultures. All the cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamate. The HL60/ADR cell line was a kind gift from Professor H.J. Broxterman (Department of Medical Oncology, Vrije Universiteit, The Netherlands) and was cultured in the continual presence of 200 ng/ml doxorubicin (Dakota Pharmaceutics, France). Cells were cultured at 37 °C in an atmosphere of 5% CO_2–95% air.

Western blots and quantification. Cells were homogenized vol:vol in RIPA buffer [PBS containing 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1 nM Na-vanadate, and 1 tablet complete cocktail protease inhibitors (Boehringer–Mannheim, Germany)]. After several passages in a 2 ml glass Potter homogenizer, the homogenates were centrifuged at 4 °C at 13,000*g* for 30 min. The resulting supernatants were assayed for protein concentration (Bio-Rad, Ivry sur Seine, France) prior to analysis under reducing conditions in 15% SDS–PAGE. Western blots were performed using standard protocol. Antibodies bound to Immobilon-P (Millipore, St. Quentin Yvelines, France) were detected by ECL (Amersham, Orsay, France) using a second antibody coupled to peroxidase. The amount of immunoreactive proteins was quantified using IP-Lab Gel Program (Signal Analytics, Vienna, USA) after scanning with an Imager (Quantum-Appligene, Illkirch, France).

Confocal microscopy. Paraformaldehyde fixed cells were incubated with TO-PRO 1 (Molecular Probes, Leiden, The Netherlands) permeabilized with 0.1% SDS and then labeled with an antibody directed against active C3 (BD Biosciences). After extensive washing, the sections were incubated for 1 h with anti-rabbit IgG coupled to Alexa 568 (Molecular Probes). All incubations were done at room temperature. The slides were mounted with mowiol and the images were visualized using a Leica TCS NT microscope with a 63×1.3 NA Fluotar objective (Leica, France).

Preparation of cytosolic extracts and analysis of caspase activity. The cytosolic extracts were prepared as described earlier [15]: briefly, cells were collected and centrifuged at 800*g* for 10 min at 4 °C. The cell pellets were washed with ice-cold PBS and then resuspended vol:vol in CEB [250 mM sucrose, 50 mM Hepes, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 1 mM EDTA, 1 mM EGTA, and 1 tablet complete cocktail inhibitor]. Cells were allowed to swell for 30 min on ice. The cells were then homogenized with 30 strokes in a 2 ml glass Potter homogenizer. The homogenates were centrifuged at 800*g* for 10 min at 4 °C and then the resulting supernatants were centrifuged for a further 15 min at 13,000*g* at 4 °C to obtain the mitochondrial pellets (P13). The supernatants were then centrifuged at 100,000*g* for 30 min in an airfuge (Beckman Instruments, France) and the resulting cytosolic fraction was further referred to as the S100. DEVDase and LEHDase activities were measured by following the degradation of fluorometric substrate in a whole cell assay or a cell free system as previously described [15,16].

Site-directed mutagenesis and transfections. Mutants of C3 were obtained by site-directed mutagenesis using the PCR based Gateway method (Invitrogen) and the amplified products were subcloned into the mammalian expression vector pDEST according to the manufacturer's instructions. Primers used for site-directed mutagenesis are listed in Table 1. MCF-7 cells with a low expression of C3 were transfected with the C3 mutants subcloned into pDEST 12.2 together with the pDsRed 1 plasmid (Clontech, Ozyme, France), a marker of

Table 1
Oligonucleotides used for site-directed mutagenesis of human C3

	Nucleotide sequence
C3 124S	GGGGACCACTTTGTACAAGAAAGCTGGGTGATGGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGC
C3 78S	GGGGACCACTTTGTACAAGAAAGCTGGGTGATGGACTCTGGAATATCCCTGGACAACAGTTATAAAATGG
C3NTM 78S	GGGGACCACTTTGTACAAGAAAGCTGGGTGATGTCCTGGGACACCGGTTATAAAATGGATTATCCTGAGATGG
C3 AS	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTTAGTGATAAAAATAGAGTTCTTTTGTGAGCATGGAAACAAT ACATGG
C3/I31M	CA ATG GAC TCT GGA ATG TCC CTG GAC
C3/L33W	CA ATG GAC TCT GGA ATA TCC TTG GAC AAC AGT TAT AAA ATG G
C3/N35T	CA ATG GAC TCT GGA ATA TCC CTG GAC ACC AGT TAT AAA ATG G
C3/S36G	GGA ATA TCC CTG GAC AAC GGT TAT AAA ATG G

C3 78S and C3NTM 78S oligonucleotides together with C3 AS were used to amplify a C3 (aa 27–280) and C3-NTM (aa 27–280) the enzyme lacking the prodomain. C3 124S was used to generate a C3 lacking the first 39 amino acids of the pC3. Oligonucleotides C3/I31M, C3/L31M, C3/L33W, C3/N35T, and C3/S36G were used with C3 AS to generate the corresponding single acid amino substitution in the wild-type C3 sequence.

the efficiency of the transfection. Plasmid DNA (5 μg) was introduced into 10^6 cells by electroporation (GenePulser, Bio-Rad) using 200 V/cm and 250 μF and the caspase activity was determined in a whole cell assay after 12 h as described [16].

Results

The apoptotic response to non-mrp substrates is altered in HL60/ADR cells

Several works have shown that the MDR phenotype was often associated with a dysfunction of the apoptotic program in leukemia cell lines [10–13]. In most cases, the expression of P-gp after treatment with a cytotoxic drug was accompanied by an inhibition of caspase activation upstream of the release of cytochrome *c* from mitochondria [11–13]. However, the influence of other proteins responsible for a MDR phenotype on the apoptotic program has been less extensively studied.

To address this question, we used HL60/ADR cells, which were selected for resistance to doxorubicin and expressed mrp [14]. The resistance to apoptosis in these cells was compared to that of the wild-type HL60 cells and to do this, we used two non-mrp substrates as cell death inducers: staurosporine (STS, 1 μg/ml), a broad inhibitor of protein kinases, and a short UVB treatment (1 min, 200 kJ/cm^2). Cell death was assayed by measuring the caspase activity (i.e., degradation of the fluorogenic peptide Ac-DEVD-AMC) or by morphological analysis of chromatin condensation and C3 activity by confocal microscopy (see Materials and methods). As illustrated in Fig. 1A, the appearance and the amount of DEVDase activity were delayed in HL60/ADR cells compared to those detected in HL60 cells during both STS- and UV-induced cell death. In addition, in both cases, the maximum caspase activity in HL60/ADR cells was always inferior to that detected in HL60 cells at the peak of apoptosis (Fig. 1A). Measurement of the release into the culture medium of the cytosolic enzyme lactate dehydrogenase (LDH) suggested that in both HL60 and HL60/ADR cells the cell death kinetics and the amplitude of the response were similar to those observed in the caspase activity assay (data not shown).

Laser confocal microscopy analyses showed condensation of the chromatin in HL60 nuclei 16 h after UV treatment and this was always associated with the presence of an active C3 in the cytosol (Fig. 1B). In HL60/ADR cells, a faint C3 staining was observed in the cytosol of some cells but most cells did not exhibit condensed chromatin at this point (Fig. 1B), suggesting that the HL60 cells were in a more advance stage of apoptosis.

Western blot analyses of the cells at the onset of apoptosis (i.e., 12 h after the addition of STS) showed the presence of the p17 subunit of C3 in HL60 cells while in HL60/ADR cells, pC3 appeared to be unaffected (Fig. 1C). Next we examined the level of other caspases and/or components involved in the execution phase of apoptosis. We found that both cell lines expressed similar amounts of initiator caspases (i.e., C8 and C9) and of the executor C6 while the level of C7 appeared to be increased in HL60/ADR (data not shown).

Lack of activation of C3 in HL60/ADR

The latter results suggested the existence in HL60/ADR cells of a mechanism preventing the activation of pC3. Two pathways are involved in the activation of C3: first, in the extrinsic pathway death receptors on the cell surface initiate the activation of C8 and second, in the mitochondrial dependent pathway requiring the release of cytochrome *c* from the mitochondria into the cytosol and the activation of C9 via the apoptosome complex. Using an in vitro assay, the addition of cytochrome *c* and dATP induced a C9-like activity (e.g., LEHDase) in HL60/ADR S100 at comparable levels to that observed in HL60 S100 (Fig. 2A). The level of C9 activity

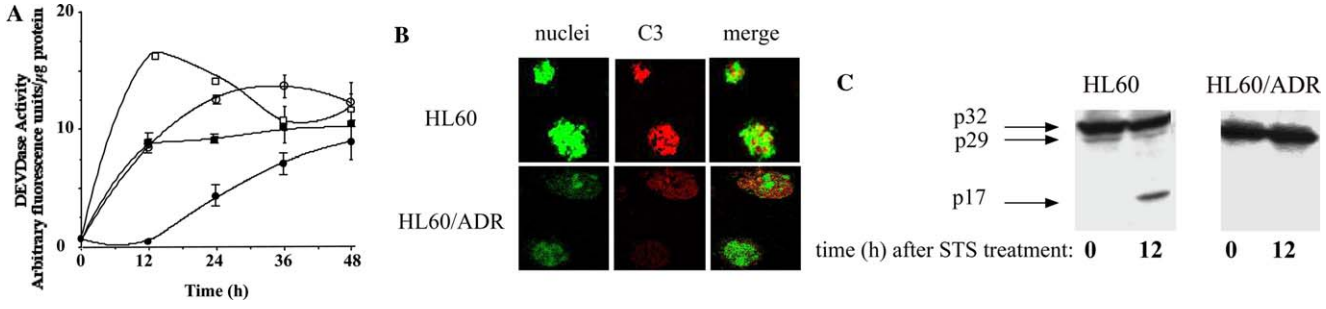

Fig. 1. The induction of apoptosis is delayed in HL60/ADR cells. (A) Kinetics of induction of DEVDase activity by UVB in HL60 (□) and HL60/ADR (○) cells or by STS in HL60 (■) and HL60/ADR (●) cells. Data shown were calculated from five different experiments (means ± SEM). (B) Confocal analysis of the nuclei (TO-PRO) and in the presence of active C3 in HL60 and HL60/ADR cells after UVB-treatment (1 min irradiation then 24 h incubation, magnification 120×). The micrographs shown are representative of five different experiments. (C) Immunoblot analysis of pC3 activation in HL60 and in HL60/ADR cells before and after the addition of STS. Fifty microgram of whole cell extracts were separated in 12% SDS–PAGE gels and then immunoblots were performed using an antibody directed against C3. The blot is representative of three different experiments.

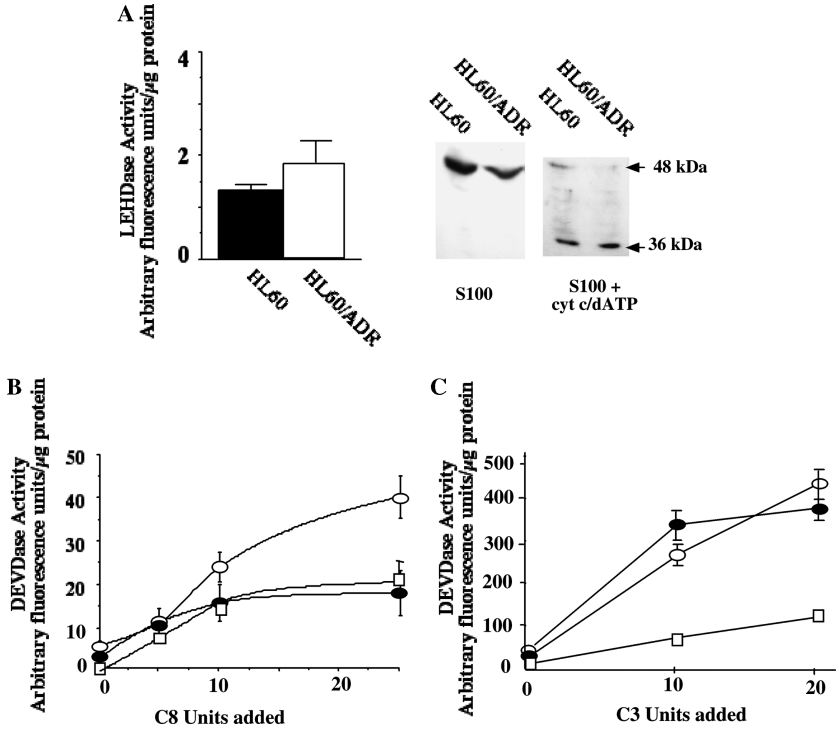

Fig. 2. Role of initiator caspases and C3 in C3-NTM activation. (A) Western blot analysis of C9 (using similar amounts of C9-like activity (namely LEHDase)) was measured in S100 (5 μg) from HL60/ADR (■) and HL60 (□) upon the addition of cytochrome *c* (cyt *c*; 20 μM) + dATP (200 μM). Data shown are means ± SEM of three different experiments. (B) Lack of activation of C3-NTM by recombinant C8 in HL60/ADR S100. 0, 10, and 20 UI recombinant C8 were added to HL60 (○) or HL60/ADR (●) S100 (5 μg). The amount of DEVDase activity in the absence of cell extracts was measured in parallel (□). Data shown are means ± SEM of three different experiments. (C) 0, 10, and 20 UI recombinant C3 were added to HL60 (○) or HL60/ADR (●) S100 (5 μg). The amount of DEVDase activity in the absence of cell extracts was measured in parallel (□). Data shown are means ± SEM of three different experiments.

suggested that the activation of this initiator caspase was not the rate-limiting step in the activation of pC3 in HL60/ADR cells. The addition of active C8 to the cell free system induced the appearance of a C3 activity in HL60 S100 but not in HL60/ADR S100 (Fig. 2B). Conversely, the addition of active C3 induced a rapid increase in DEVDase activity in both HL60 and HL60/ADR S100 (Fig. 2C).

Mutations in the N-terminus of the p17 subunit impaired the correct activation of C3 in HL60/ADR cells

Our previous results have shown that pC3 could not be activated in HL60/ADR S100. We isolated by RT-PCR, the cDNAs encoding for pC3 in HL60 and HL60/ADR cells. A single band of 839 bp was obtained for both HL60/ADR and HL60 cells (data not shown). As

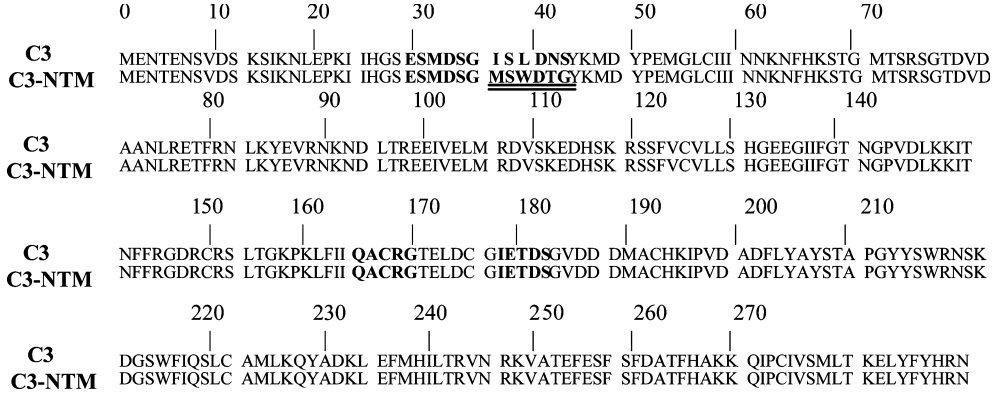

Fig. 3. A sequence located at the N-terminus in C3 from HL60 differs from that of C3 present in HL60/ADR. Predicted amino acid sequence of the C3-NTM deduced from the cDNA sequence of HL60/ADR C3 and its alignment with wild-type C3 obtained from HL60 cells. The C3-NTM sequences in HL60 and the corresponding sequence in HL60/ADR are highlighted.

shown in Fig. 3, the protein sequence deduced from the cDNA that was obtained from HL60 cells was identical to the published human sequence [17]. The sequence obtained from HL60/ADR cells (EMBL nucleotide sequence database Accession No. AJ413269) contained minor changes in the sequence in the N-terminal of the p17 subunit implying a modification immediately after the site of cleavage ESMD (Fig. 3). We have called this form of pC3 the N-terminal modified C3 (pC3-NTM). As illustrated in Fig. 3, the sequence **GISLDNSY** (aa 30–37) in the wild-type human C3 sequence was substituted by the following sequence **GMSWDTGY** in C3-NTM.

To confirm the importance of this mutation in pC3 activation we expressed transgenes encoding for the full length pC3 and C3-NTM in the MCF-7 cell line, taking advantage of the low expression of endogenous pC3 in these cells (data not shown). As shown in Fig. 4, no caspase activity was detected upon the transient expression of both pC3 and pC3-NTM zymogens. On the

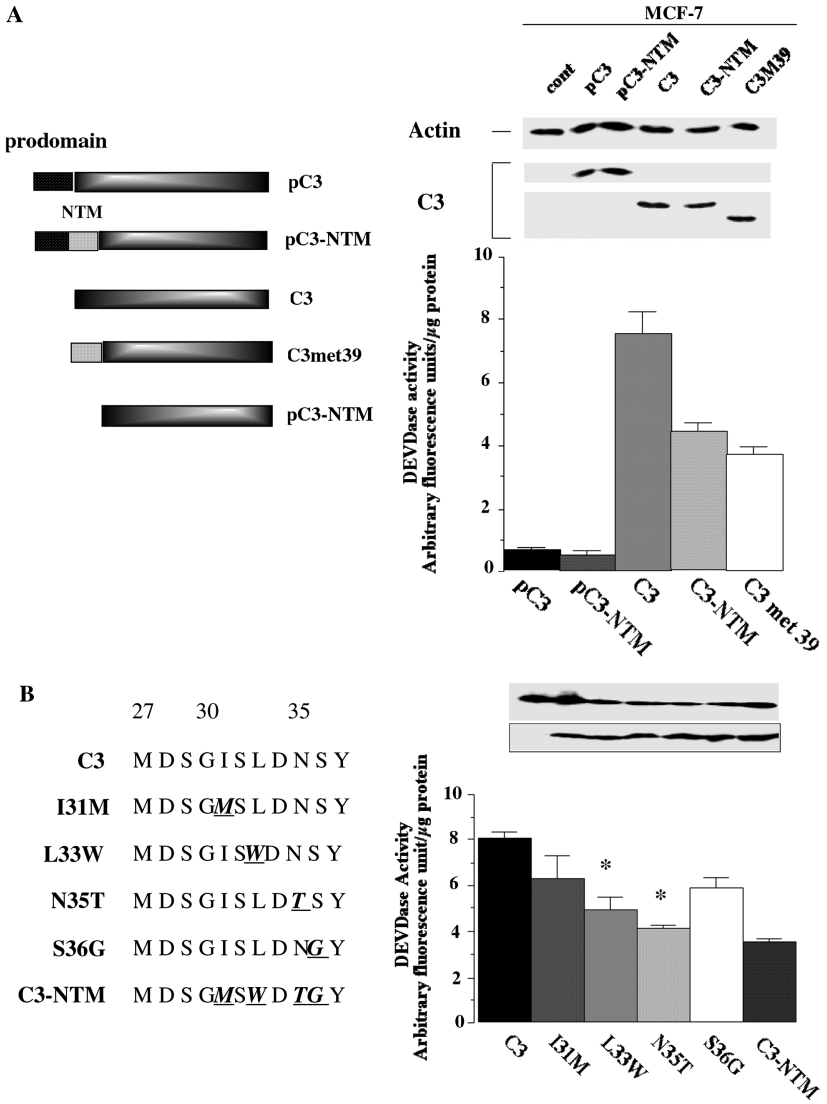

Fig. 4. A sequence located in the N-terminus of C3 is involved in the control of its activation. (A) Expression of transgenes encoding for C3 and C3-NTM constructs. 10^6 MCF-7 cells were transiently transfected with cDNA (1–5 μg) encoding for the pC3, the pC3-NTM, the C3 sequence without its prodomain (C3), the pC3-NTM sequence without its prodomain (C3-NTM), and a construct in which the first 38 amino acids of C3 were deleted (C3M39). Immunoblots using 50 μg cell extracts show that equivalent amounts of C3 were produced for each construct. The amount of DEVDase activity was measured after 12 h, which corresponded to the peak of activity produced by C3 (data not shown). Data shown are means ± SEM of at least three different experiments. Left: schematic representation of the C3 constructs. (B) Single amino acid substitution of the NTM sequence in wild-type C3. To evaluate the importance of each amino acid substitution in the NTM sequence, constructs in which these amino acids were singly substituted in the wild-type sequence were obtained and expressed in MCF-7 as described in (A). Immunoblots using 50 μg cell extracts show that equivalent amounts of C3 were produced for each construct. Data shown are means ± SEM of at least three different experiments. Left: amino acid sequences of the constructs used.

other hand, transfection of the mature C3 (i.e., without the prodomain) induced a rapid appearance of a DEVDase activity (Fig. 4). The expression of the C3-NTM was also accompanied by the appearance of a DEVDase activity although this activity was significantly and considerably reduced by half ($P = 0.001$). Similarly, a deletion of the first 38 amino acids of C3 reduced the DEVDase activity in the MCF-7 cells ($P = 0.001$). Since the level of transfection was similar in all cases as judged by the co-transfection with the pDsRed1 plasmid, we concluded that the mutations observed in C3-NTM or that deletion of this sequence drastically affected the appearance of C3 activity. To determine the nature of the residues involved in the inhibition of the activation of C3-NTM, we generated a series of C3 mutants in which amino acids were singly substituted by the corresponding amino acid modified in pC3-NTM (Fig. 3). Each construct was transiently expressed in MCF-7 and the spontaneous DEVDase activity was measured as described above. As shown in Fig. 4, the I31M substitution had no effect on the DEVDase activity induced by the expression of the transgenes ($P = 0.0627$). On the other hand, the DEVDase activity was significantly decreased by the substitution of a Leu for a Trp at position 33 (L33W, $P = 0.0133$), by a Ser for a Gly at position 36 (S36G, $P = 0.0133$) or by that of an Asn for a Thr at position 35 (N35T, $P = 0.003$). These results suggested that the two residues flanking Asp-34 were determinant in C3 activation.

Discussion

We and others have recently shown that an acquired MDR was usually associated with a inhibition or a delay in the activation of caspases [11–13]. An analysis of the apoptotic program in the mrp-expressing cell line, HL60/ADR, suggested an impediment to cell death downstream of the transmembrane transporter. We have shown that the induction of a C3-like activity is delayed in HL60/ADR cells (Fig. 1). The sequence of the cDNA encoding for C3 in HL60/ADR cells revealed a change in the N-terminal sequence in the large subunit immediately after the N-peptide (Fig. 2). The length and the nature of the prodomains differ between the different groups of caspases. The initiator caspases (e.g., C8, C9) have long prodomains (N-peptides), which include the death effector domains or caspase recruitment domains necessary to form the caspase activator complexes. Once the activator complexes are formed, the initiator caspases acquire catalytic activity and may activate executioner caspases (C3, C7) by direct limited proteolysis. These executioner caspases have short prodomains without any recognizable folding pattern. Meergans et al. [18] have shown that the removal of the C3 prodomain provoked a spontaneous activation of the protein when expressed in HeLa cells and we confirmed this result in MCF-7 cells (Fig. 3). These findings suggest that the short prodomain of C3 serves as a silencing component in mammalian cells by retaining this executioner caspase in an inactive state. A similar observation was made for C7 [19]. However, in this paper we show that a sequence located immediately after the prodomain could also be involved in this silencing. We provide several lines of evidence as specific mutations or deletions of this domain impaired the spontaneous activity of C3 in MCF-7 (Fig. 4). The fact that this domain controls the activation of C3 by initiator caspases such as C8 or C9 but not by C3 itself (Fig. 2) is quite intriguing as the cleavage of the ESMD site is thought to be an autocatalytic process while that of the initiator caspases occurs at a more distant site (viz. IETD). C3 activation, both in vitro and in vivo, has been shown to occur by interdomain cleavage followed by removal of the prodomain [20]. The importance of both amino acids flanking D34 on the inhibitory effect in pC3-NTM activation suggests that these residues play an essential role in pC3-NTM function (Fig. 4). One possibility is that pC3-NTM is a non-canonical site for initiator caspases and thus is cleaved upon induction of apoptosis. Indeed, the presence of a p29/C3 is quite often observed in HL60/ADR cell extracts (data not shown). A form of pC3 truncation has been detected in cells after calpain activation [21]. In addition, this calpain processing of pC3 has been shown to be associated with the inability of C8 or C9 to activate C3. It is also conceivable that the NTM sequence controls the structure of C3 through correct folding or dimerization, a function inhibited by the prodomain in many zymogens [22]. Of note, Mittl et al. [23] studying the 3D structure of pC3 showed that the sequence between S29 and N35 (encompassing the NTM sequence) could not be defined by electron density, indicating that this sequence was highly disordered.

The fact that activation of C3-NTM was completely inhibited in K562/ADR cells while this inhibition was only partial in MCF-7 suggests that additional elements interfering with pC3 activation are also present in the MRP-expressing cell line. In summary, we have identified an unexpected sequence involved in the mechanism of caspase activation. Recently, Roy et al. [24] have identified, downstream of the IETD site, a regulatory acidic tripeptide (Asp-Asp-Asp), which is also involved in the control of pC3 activation. They have called the yet unidentified mechanism by which this tripeptide regulates C3 activation a "safety catch." Removal of this safety catch enhanced the sensitivity of activation of pC3 by other caspases. We believe that amino acids 31 to 36 could represent another "safety catch," which could also regulate the "proper" activation of C3 by a mechanism which remains to be defined.

Acknowledgments

We thank Professor H.J. Broxterman (Department of Medical Oncology, Vrije University, The Netherlands) for the gift of the HL60/ADR cell line. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale. M.P. and P.F.C. were supported from a fellowship from the Ligue contre le Cancer.

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