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Apaf-1XL Is an Inactive Isoform Compared with Apaf-1L

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Apaf-1 plays a crucial role in the cytochrome *c*/dATP-dependent activation of caspase-9 and -3. We found that the human myeloid leukemic K562 cells were more resistant to cytochrome *c*-induced activation of caspase-9 and -3 in a cell-free system compared with the human T-lymphoblastic subclone CEM/VLB_{100} cells. Apaf-1 cDNA sequencing revealed an additional insert of 11 aa between the CARD and CED-4 (ATPase) domains in K562 cells, which was identical to the sequence of Apaf-1XL. Immunoprecipitation of Apaf-1 with caspase-9 after a cell-free reaction demonstrated that Apaf-1XL in the K562 cell line showed a lower binding ability to caspase-9 compared with Apaf-1L protein. The resistance of K562 cells to cytochrome *c*-dependent apoptosis may be partly due to this Apaf-1XL form. These results suggest that the additional insert between CARD and CED-4 domains might affect Apaf-1 recruitment of caspase-9 during apoptosis.

Key Words: **Apaf-1; apoptosis; caspases; cytochrome *c*; cDNA cloning; leukemia.**

Resistance to apoptosis has been implicated as one potential mechanism whereby tumor cells evade chemotherapeutic drug and immune-mediated destruction. For the majority of anticancer drugs, apoptosis appears to be initiated by the cytochrome *c*/Apaf-1/caspase-9 pathway (1, 2). Failure to activate the apoptotic machinery can result in resistance to the cytotoxic effects of multiple chemotherapeutic agents (3–6). The Apaf-1 cytochrome *c* complex recruits procaspase-9 in a dATP/ATP dependent manner through a CARD-CARD interaction, resulting in its activation with the presumed release of mature caspase-9 from the apoptosome (7–10). Once bound to Apaf-1, procaspase-9 will be activated by cytochrome *c* and dATP, inducing the activation of caspase-3 and leading to apoptosis (8, 9, 11). The activation of the cytochrome *c*/Apaf-1/caspase-9 pathway depends on the protein Apaf-1 (12, 13).

Apaf-1 cDNA, which encodes 1194 amino acids (aa), was first cloned from the HeLa cell line in 1997 by Xiaodong Wang's group (14). This 130-kD protein comprises of three functional domains, the N-terminal CARD (caspase recruitment domain) followed by a putative ATPase domain or CED-4 and a C-terminal domain with a 12 WD-40 repeat region, which functions as a negative regulatory element of Apaf-1 (15–19). Five types of Apaf-1 cDNA have been identified in human tissues. In addition to the original Apaf-1, the Apaf-1L and Apaf-1XL isoforms have 13 WD-40 repeats and Apaf-1XL also has an insertion between CARD and ATPase domains (8, 9, 12, 20). Apaf-1M contains an insert after CARD but has 12 WD-40 repeats (20, 21). Apaf-1XS contains an insert between CARD and ATPase, but it has a deletion in the WD-40 repeat region (20).

The important role of Apaf-1 in the regulation of apoptosis has been revealed by analysis of mutant mice deficient in Apaf-1. Mice lacking Apaf-1 showed resistance to apoptosis induced by several stimuli (22, 23). We have previously shown that the human leukemic K562 cell line was resistant to UV-light induced apoptosis, due to lower levels of Apaf-1 protein compared with the human T-lymphoblastic leukemic CEM/VLB_{100} cell line which is sensitive to UV light-induced apoptosis (5). In this study, we demonstrated that the lower levels of Apaf-1 in K562 cells was due to a failure in binding of the monoclonal Apaf-1 antibody to Apaf-1XL protein. Apaf-1XL isoform displayed lower ability to recruit procaspase-9 compared with Apaf-1L in human leukemic cells.

MATERIALS AND METHODS

Materials. Two monoclonal anti-Apaf-1 antibodies were used, one was kindly provided by Cold Spring Harbour Laboratory (CSHL, 24) and another Apaf-1 antibody, clone 24 was obtained from Transduction Laboratory-BD Company (Lexington, KY). Polyclonal anti-Apaf-1 antibody (NT) was bought from QED Bioscience Inc. (Calne, UK). Monoclonal mouse anti-casepase-9 antibodies, clone 5B4 and clone 2-23 were obtained from StressGen-Bioquote Ltd. (North York-

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shire, UK). Total RNA extraction reagent and RT-PCR kits were from Gibco BRL (West Sussex, UK). Gel and plasmid extraction kits were purchased from QIAGEN Ltd. (West Sussex, UK). PGEM-T Easy Vector and JM109 competent cells were from Promega (Southampton, UK). Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC) and Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC) were from Calbiochem (Nottingham, UK). Primers were synthesised by Cambridge BioScience Ltd (Cambridge, UK). All chemicals used in this study were obtained from Sigma (Dorset, UK).

Cell lines, cell culture and AML blasts. The human erythroleukemic K562 cell line and T-lymphoblastic leukemia CEM/VLB$_{100}$ cell line, which is a vinblastine-resistant subclone, were used in this study. These cell lines were grown in RPMI 1640 with 10% (v/v) FCS as described previously (25). Human AML blasts were obtained from patients presenting with acute myeloid leukemia and were separated over a Ficoll gradient (Amersham Pharmacia Biotech, Sweden).

Preparation of S-100 and a cell-free reaction. Leukemia cells (5×10^7) were washed with Ca^{2+} and Mg^{2+}-free PBS and resuspended in 1 ml of Buffer A (250 mM Sucrose, 10 mM Hepes-KOH, pH 7.4, 10 mM KCL, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μM cytochalasin B) and incubated on ice for 30 min. Cells were then broken on ice with a glass Dounce homogeniser (Jencons, Leighton Buzzard, UK). The homogenate was centrifuged at 12,000*g* for 40 min at 4°C. The supernatant was then transferred to a new tube with 0.22 micro nylon membrane (Sigma) and centrifuged at 12,000*g* for 40 min at 4°C. Protein concentration was measured by a Bio-Rad reagent (Bio-Rad). For a 25 μl cell-free reaction system, 50 μg protein containing S-100 in Buffer A was incubated with or without cytochrome *c* (50 ng)/dATP (10 nM) at 30°C for 15 min or 30 min. 5 μl of fluorogenic substrate working solution (400 μM), Ac-LEHD-AFC for caspase-9 or Ac-DEVD-AFC for caspase-3 was added to the reaction system and further incubated at 30°C for 15 min. The reaction was stopped by the addition of 50 μl of 1% sodium acetate trihydrate in 175 mM acetic acid. The casepase-9 or -3 activity was measured as described previously (5).

Expression of Apaf-1 cDNA. Total RNA was prepared using TRIzol Reagent and cDNA was synthesised with THERMOSCRIPT reverse transcriptase and Oligo $(dT)_{20}$ primer. One pair of primers overlapping the CARD and ATPase domains of Apaf-1 was designed as follows: 5′-CGG AAT TCG ATG GAT GCA AAA GCT CG-3′ and 5′-ACT GAC TGC ACA ATC CTT TTC-3′ (1-1590). β-actin (5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′) was used as an internal control. Two pairs of primers were added in a 50 μl of PCR reaction system with 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 90 s, followed by extension at 72°C for 10 min. After separated in 1% agarose gel, PCR products were analyzed with an AlphaImager 2000 Densitometer (Alpha Innotech Corp., San Jose, CA).

Apaf-1 cDNA sequencing. Total RNA extraction and the cDNA synthesis were same as above. Four pairs of Apaf-1 cDNA fragments were amplified with the following primers: 5′-ATG GAT GCA AAA GCT CGA AAT T-3′ and 5′-GGC ACC TTA ACG TCC TTC TG-3′ (1-1154); 5′-TGA GGC TCT AGA TGA AGC CA-3′ and 5′-CCC AAA GTT TGA GGA AGC AG-3′ (1050-2152); 5′-AGT CAA TTG CTG CCA TTT CA-3′ and 5′-TTG TGC TGA AAC CTG GAC TG-3′ (2070-2975); 5′-GGA TCA TCA TTT TTG ACA TCT TC-3′ and 5′-AAA TAC CAA GAT TAT CCA CAG TCA CA-3′ (2647-3688) by PCR. After denatured at 94°C for 3 min, cDNAs underwent 35 cycles of denaturization at 94°C for 45 s, annealing at 58–59°C for 45 s, and extension at 72°C for 70 s. The additional extension was at 72°C for 10 min. After the PCR products were separated in 1% low melting agarose gel, the target bands were cut with clean scalpel blades and purified with QIAquick Gel Extraction Kit. Purified PCR products were then cloned into pGEM-T Easy Vector. Positive clones were selected by β-galactosidase screening system. After amplification and extraction, plasmids from the positive clones were identified with restriction enzymes *Nco*I and *Pst*I. Apaf-1 PCR fragments in plasmids were sequenced with T20/SP6 primers by Cambridge BioScience Ltd. and analyzed using Blast 2 sequences programme (NCBI).

Immunoprecipitation and Western blotting. 500 μg protein containing S-100 in 250 μl Buffer A was incubated with or without cytochrome *c* (50 μg/ml)/dATP (10 nM) at 30°C for 15 or 30 min. 13 μl of anti-caspase-9 antibody (clone 2-23) was added to the cell-free reaction and incubated for 2 h at 4°C on a rotor. 25 μl of Dynal M-450 beads (1×10^7) (Dynal, UK) was then added to the reaction system to bind the complex of caspase-9 and anti-caspase-9 antibody, and incubated at 4°C on the rotor overnight. After rinse 4 times with Buffer A, beads were collected with a Dynal Magnetic Particle Concentrator (Dynal). Bound Apaf-1 and caspase-9 were eluted with 25 μl of sample buffer for Western blotting.

For Western blotting (4), the protein sample was separated in 8% or 10% SDS-PAGE at 10–15 mA/gel and transferred onto a PVDF membrane (Sigma) for 1–2 h. The blot was blocked by 1% non-fat milk and probed with antibody (as described individually in figure legends). Bound antibodies were detected using appropriate HRP-conjugated secondary antibodies, followed by detection using Super-Signal enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Rainbow markers (Amersham, Little Chalfont, UK) served as standard molecular weights. The density of each band was analyzed using an AlphaImager 2000 Densitometer (Alpha Innotech Corp., San Jose, CA).

RESULTS AND DISCUSSION

Expression of Apaf-1 Protein and cDNA

We have previously shown that the K562 cell line was more resistant to UV light-induced apoptosis as it had lower levels of Apaf-1 protein when compared with CEM/VLB$_{100}$ cells (5). We initially detected differential levels of Apaf-1 protein expression in K562 and CEM/VLB$_{100}$ cell lines using a mouse monoclonal anti-Apaf-1 antibody, provided by the Cold Spring Harbour Laboratory (CSHL, 24). The ratio of Apaf-1 protein levels in K562 to CEM/VLB$_{100}$ cells was 1:4 (5; Fig. 1A). To more precisely evaluate the expression of Apaf-1 protein, two monoclonal mouse anti-Apaf-1 antibodies and one polyclonal rabbit anti-Apaf-1 antibody immunogenised from different regions of Apaf-1 were used in this study. The differential Apaf-1 levels in K562 and CEM/VLB$_{100}$ cells (1:4) were confirmed by another monoclonal Apaf-1 antibody produced by Transduction Laboratory (BD). However, there was no difference in Apaf-1 protein levels between the two cell lines when detected using a polyclonal rabbit Apaf-1 antibody from QED Bioscience Inc. (Fig. 1A). We also confirmed this finding of similar levels of Apaf-1 protein between the two cell lines with another polyclonal Apaf-1 antibody obtained on this occasion from Alexis Biochemicals Corp (data not shown). As shown in Fig. 1B, the monoclonal mouse anti-Apaf-1 antibody was immunogenised against N-terminal long fragments as antigens. The Apaf-1 antibody from CSHL was raised against a 50-kDa N-terminal fragment of the protein (24) and the antibody from the Transduction Labo-

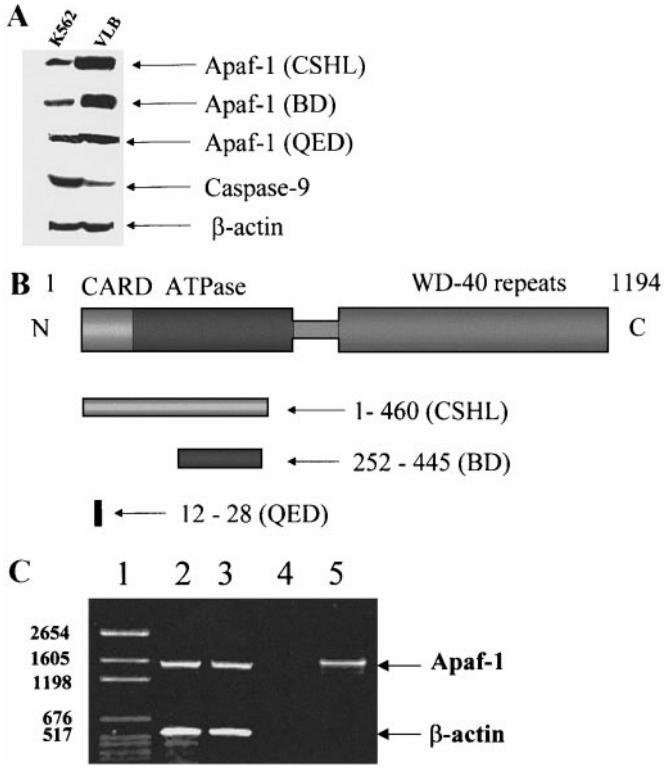

FIG. 1. Expression of Apaf-1 protein in leukemic cell lines. (A) Expression of Apaf-1 protein in K562 and CEM/VLB$_{100}$ cell lines. 200 μg, 50 μg or 25 μg of protein lysis was analyzed by Western blot using Apaf-1 antibody from Cold Spring Harbour Laboratory (CSHL), Transduction Laboratories (BD) or QED Bioscience Inc. (QED), respectively. The dilution of Apaf-1 antibody was 1:1000 (CSHL), 1:250 (BD) or 1:1000 (QED). Caspase-9 expression was performed on 50 μg protein and detected by monoclonal mouse caspase-9 antibody (clone 5B4) at 1:1000 dilution. β-actin used as the loading control. 25 μg protein was probed by a monoclonal β-actin antibody at 1:10,000 dilution. (B) Illustration of immunogen regions of Apaf-1 antibodies. Numbers indicated are positions of amino acids. (C) Expression of Apaf-1 cDNA in K562 and CEM/VLB$_{100}$ cell lines. Lanes 1–5 indicate pGEM DNA markers, the K562 cell line, CEM/VLB$_{100}$ cell line, negative control (no template) and positive control (plasmid containing Apaf-1 cDNA as template), respectively.

ratory (BD) was immunised using the N-terminal 252-445 aa as an immunogen. However, the polyclonal Apaf-1 antibody from QED was prepared using a small N-terminal peptide corresponding to 12-28 aa fragment of human Apaf-1. Using RT-PCR, we confirmed that Apaf-1 mRNA levels in K562 and CEM/VLB$_{100}$ cells were also similar, as the ratio of Apaf-1 to β-actin in the K562 and CEM/VLB$_{100}$ was 46:54 (Fig. 1C). These results imply that the actual Apaf-1 protein levels between K562 and CEM/VLB$_{100}$ cell lines could be similar. The lower levels of Apaf-1 protein in K562 cells as detected by the monoclonal antibody may be due to differences in protein structure or protein folding which affected detection by the monoclonal mouse Apaf-1 antibody.

K562 Cells Were More Resistant to Cytochrome c-Induced Activation of Caspase-9 and -3 Compared with CEM/VLB$_{100}$ Cells

A cell-free system for Apaf-1/cytochrome *c*-dependent activation of caspases was designed to test the function of Apaf-1 protein in K562 and CEM/VLB$_{100}$ cell line. S-100 was extracted from the two cell lines. The reaction was initiated by addition of cytochrome *c* and dATP to the S-100. Caspase-9 and -3 activities were measured by the cleavage of fluorogenic substrates, Ac-LEHD-AFC for caspase-9 or Ac-DEVD-AFC for caspase-3. The K562 cell line showed significant resistance to cytochrome *c*-induced activation of caspase-9 (Fig. 2A) and caspase-3 (Fig. 2B) compared with CEM/VLB$_{100}$ cells, as analyzed by the one-way ANOVA ($P < 0.001$). As shown in Fig. 1A, K562 cells expressed higher level of procaspase-9 compared with CEM/VLB$_{100}$. We also detected that the negative factors of the apoptosome, such as XIAP (26) or caspase-9b (27), were expressed at a similar levels between two cell lines (5). Transfection of Apaf-1L gene increased the sensitivity of K562 cells to cytochrome *c*-dependent apoptosis. The ability of transfected Apaf-1L protein to recruit procaspase-9 was not affected in the K562 cells (5). We suggest that Apaf-1 protein in these two cell lines might be different in both function and structure.

Apaf-1 in K562 Cells Contained an Insert between CARD and ATPase Domains

We therefore decided to investigate whether there was a difference in Apaf-1 cDNA sequences between the K562 and CEM/VLB$_{100}$ cell lines. Apaf-1 cDNA clones from K562 and CEM/VLB$_{100}$ cell lines were obtained by RT-PCR. Sequencing and computational an-

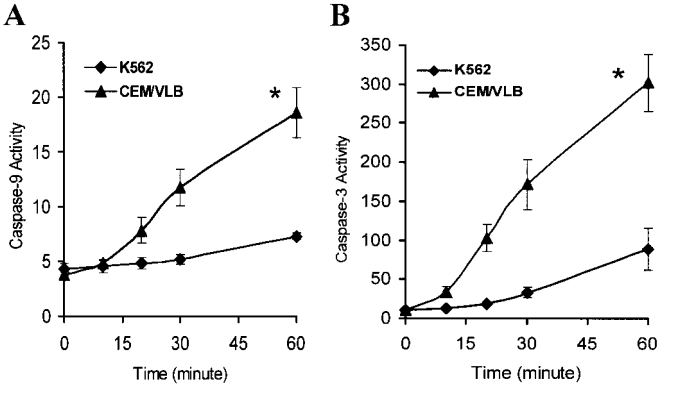

FIG. 2. Cytochrome *c*-mediated activation of caspases. S-100 was extracted and purified from K562 and CEM/VLB$_{100}$ cell line. 50 μg of protein was used for each reaction. Cytochrome *c*-mediated time-dependent activation of caspases-9 (A) and caspases-3 (B) were evaluated by the release of AFC from their fluorogenic substrates. Activity of caspases was defined as μM AFC release per h of 1 mg protein (μM/hr/mg). Significant difference (*$P < 0.001$) in activity between two cell lines was analyzed by ANOVA.

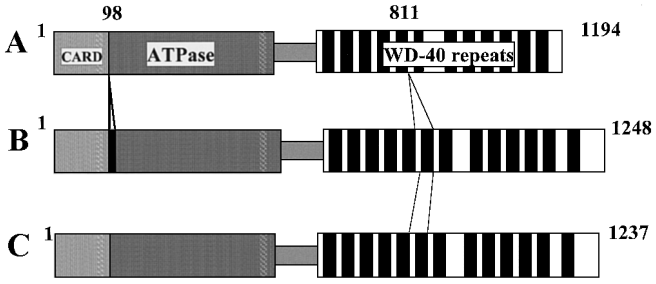

FIG. 3. Schematic representation of Apaf-1 forms. (A) The original Apaf-1 (4). (B) The Apaf-1XL from the K562 cell line. (C) The Apaf-1L from the CEM/VLB$_{100}$ cell line.

alyzes using the BLAST programme revealed differential Apaf-1 cDNA sequence in K562 and CEM/VLB$_{100}$ cell lines compared with the original Apaf-1 (Fig. 3). An additional fragment of 33 nucleotides between the CARD and ATPase domains of Apaf-1 cDNA was inserted in the Apaf-1 cDNA of the K562 cell line. The sequence of the insertion is identical to those published by other groups (1, 8, 12, 21). The Apaf-1 sequence in both K562 and CEM/VLB$_{100}$ cells contained 13 WD-40 repeats compared with the original Apaf-1, which contains 12 WD-40 repeats (14). The Apaf-1 cDNA from the K562 cell line was identical to Apaf-1XL (8) and that from the CEM/VLB$_{100}$ cell line revealed the similar sequence to the Apaf-1L (9).

To confirm whether Apaf-1 antibodies employed in this study could evaluate Apaf-1L protein levels correctly, two human AML patient blast samples were used to analyze Apaf-1 cDNA sequence and Apaf-1 protein expression. Apaf-1 cDNA sequence in both AML blasts was identical to the Apaf-1L (data not shown). However, they expressed remarkably different levels of Apaf-1 protein no matter which kind of Apaf-1 antibody was used for the detection (Fig. 4). The ratios of Apaf-1 protein expression in AML-1 to AML-2 were similar when using either a monoclonal Apaf-1 antibody from Transduction Laboratory (BD) or a polyclonal Apaf-1 antibody from QED. These two antibodies could detect Apaf-1 protein levels correctly if there is no additional insert between CARD and ATPase. We also found that AML-1 blasts were extremely resistant

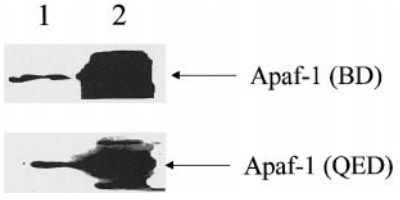

FIG. 4. Expression of Apaf-1 protein in AML blasts. 50 μg protein was used for Apaf-1 expression. Monoclonal anti-Apaf-1 antibody from BD was used at 1:250 dilution. Polyclonal anti-Apaf-1 antibody from QED was used at 1:1000 dilution. Numbers indicated are 1, AML-1; 2, AML-2.

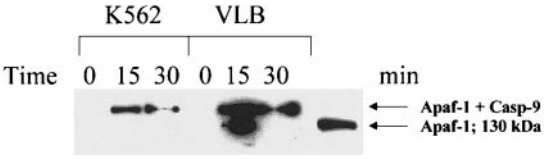

FIG. 5. Apaf-1XL showed lower binding ability to caspase-9. S-100 (500 μg protein) extracted from K562 and CEM/VLB$_{100}$ (indicated as VLB) was incubated with cytochrome *c*/dATP for 15 or 30 min. 25 μl of DYNAL beads (1×10^7) and 3 μg of mouse anti-caspase-9 antibody (clone 2-23) in a 250 μl volume were used for immunoprecipitation. The rabbit anti-Apaf-1 antibody (1:1000, QED) was used to detect the precipitate. The band on the right side indicates the Apaf-1 protein control (130 kDa) from CEM/VLB$_{100}$ cell lysate (6 μg protein). The precipitates are Apaf-1/caspase-9 complex (Apaf-1 + Casp-9) and Apaf-1 protein.

to UV light-induced apoptosis compared with AML-2 blasts (5). This indicates that sufficient Apaf-1 protein levels are essential for UV light-induced apoptosis.

K562 and CEM/VLB$_{100}$ cells have similar levels of Apaf-1 protein but different sensitivity to cytochrome *c*-induced activation of caspases. We reasoned that the differential in protein structure, *e.g.*, an insert between CARD and ATPase domains in K562 Apaf-1 cDNA might affect the folding of Apaf-1 protein or lead to a decrease in its ability to recruit procaspase-9.

Apaf-1 Protein in K562 Cells Showed Lower Ability to Recruit Procaspase-9

In order to compare the binding ability of Apaf-1 to procaspase-9 between the K562 and CEM/VLB$_{100}$ cell lines, immunoprecipitation of Apaf-1 with anti-caspase-9 antibody was performed before and after the addition of cytochrome *c*/dATP in a cell-free system. Apaf-1 protein, which was precipitated with caspase-9 antibody, was detected with a polyclonal rabbit anti-Apaf-1 antibody from QED. No Apaf-1 was detected before adding cytochrome *c* and dATP (Fig. 5). Apaf-1 protein precipitated by caspases-9 antibody was shown at 15 and 30 min after addition of cytochrome *c*/dATP into a cell-free system. By comparison with standard Apaf-1 protein (130-kDa), the molecular weight of precipitated Apaf-1 protein was higher than 130-kDa. Re-probing the blot with anti-caspase-9 antibody demonstrated that these bands were a complex of Apaf-1/procaspase-9 proteins (data not shown). However, immunoprecipitated Apaf-1 in the CEM/VLB$_{100}$ cell line showed both Apaf-1/caspase-9 complex and pure Apaf-1 bands. The ability of Apaf-1 recruiting caspase-9 in the CEM/VLB$_{100}$ cell line was much greater than that in K562 cells and the ratio approximated 4:1 when caspase activation was initiated by cytochrome *c* and dATP for 15 min. The amount of Apaf-1 bound to caspase-9 did not increase with time; by contrast, it decreased in the CEM/VLB$_{100}$ cell line after 30 min reaction. These results suggested that Apaf-1 protein with an insert between CARD and ATPase domains might affect the binding ability to caspase-9.

The Apaf-1XL has been shown to have more effect on cytochrome *c*- and dATP-dependent procaspase-9 activation than the original Apaf-1 (8, 12) and it has been found that the cytochrome *c*- and dATP-dependent procaspase-9 activation by the product of the original Apaf-1 was almost undetectable (8). In this study, we show Apaf-1XL has lower ability to activate procaspase-9 and -3 in response to cytochrome *c*/dATP compared with Apaf-1L. This is due to lower binding ability of Apaf-1XL to procaspase-9. The additional 11 aa insertion between CARD and ATPase domains may affect Apaf-1XL to recruit procaspase-9 and also affect monoclonal Apaf-1 antibody to detect Apaf-1 protein expression. Further evaluation for the association of the Apaf-1XL protein conformation with its function is required to address its effect on the resistance to cytochrome *c*-dependent apoptosis.

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