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p62, a Phosphotyrosine Independent Ligand of SH2 Domain of p56^{lck}, is Cleaved by Caspase-3 during Apoptosis in Jurkat Cells

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p62 is a phosphotyrosine-independent ligand of the SH2 domain of p56^{lck}, a T-cell specific Src family tyrosine kinase. Recently p62 has been shown to interact with a number of proteins, such as PKCζ and ubiquitin, and implicated in important cellular functions such as cell proliferation. Since the two p62 interacting proteins, p56^{lck} and PKCζ, have been reported to play roles in cell death, I have addressed the potential role of p62 during apoptosis in Jurkat cells in this study. Herein I show that p62 was specifically cleaved into two peptides by a caspase-3-like activity during Fas-receptor mediated apoptosis in Jurkat cells. This cleavage generated two fragments with molecular weights of about 35 kDa that differed in subcellular localizations. The N-terminal cleaved fragment was present in the detergent-insoluble fraction whereas the C-terminal fragment was found in the detergent-soluble fraction. In addition, the C-terminal fragment appeared to be subjected to further degradation as apoptosis prolonged. Moreover, overexpression of p62 in Jurkat cells attenuated the Fas receptor mediated apoptosis, suggesting that p62 is involved in apoptotic signal transduction pathway in lymphocytes.

The putative signaling molecule p62 was originally identified as a phosphotyrosine-independent ligand of the SH2 domain of p56^{lck}, a T-cell specific Src family tyrosine kinase (Joung et al., 1996). p62 is a ubiquitously expressed phosphoprotein encoded by an immediate-early response gene that is rapidly induced by a variety of extracellular signals known to stimulate cell proliferation, differentiation, and response to oxidative stress (Ishii et al., 1996; Lee et al., 1998). p62 has a Zinc finger motif and two PEST sequences, and shares YXDED motif with several proteins involved in cell signaling such as MEK5, scd1, and cdc24. It has been also observed that p62 interacts with a number of proteins involved in important cellular functions including cell proliferation and survival, such as ubiquitin (Vadlamudi et al., 1996), orphan nuclear receptor COUP-TFII (Marcus et al., 1996), PKCζ (Pulse et al., 1997; Sanchez et al., 1998), and p38 MAP kinase (Sudo et al., 2000).

Recently several studies suggested that p62 functioned as an adaptor connecting key players in various signal transduction pathways. p62 has been implicated in NF-kB activation by tumor necrosis factor α, through linking atypical PKCs to RIP, a death domain kinase that associates with the TNF receptor I (TNF-R1) (Sanz et al., 2000). It was also reported that p62 acted as a p38 MAP kinase regulator in HeLa cells (Sudo et al., 2000). In addition, it has been shown that p62 is a major constituent in intracytoplasmic hyalin bodies (IHBs) that resembles inclusions in hepatocarcinoma cells, suggesting the involvement of p62 in carcinogenesis through linking ubiquitinated species to other proteins (Stumptner et al., 1999). Although considerable knowledge regarding roles of p62 in several cell types has been accumulated during the past few years, the biological function of p62 in lymphocytes is yet to be determined.

Among proteins that are known to associate with p62, p56^{lck} and PKCζ have been implicated in the regulation of apopotsis. p56^{lck} activity was required in apoptosis induced by ionizing radiation or ceramide treatment in Jurkat cells (Di Soma et al., 1995; Belka et al., 1999; Manna et al., 2000). It was also shown that p56^{lck} was necessary for transducing apoptotic signals mediated by the engagement of T cell receptor in mature cycling cells (Gonzalez-Garcia et al., 1997). In addition, it was reported that overexpression of PKCζ inhibited UV-induced apoptosis in NIH 3T3 cells, where it acted as a pro-survival enzyme that needed to be blocked for apoptosis to proceed (Berra et al., 1997). The association of p62 with PKCζ suggests that

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p62 plays a role in the cascade controlling cell death, perhaps by serving as a scaffold linking distinct signaling molecules. In the study reported here I have addressed the potential role and fate of p62 during apoptosis in Jurkat cells.

Materials and Methods

Cells and reagents

Jurkat leukemic T cells were maintained in RPMI 1640 medium containing 10% fetal calf serum and 100 units/ml penicillin/streptomycin. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin were obtained from Sigma. Caspase-3 inhibitor Z-DEVD-FMK was purchased from Calbiochem. Antibody against p62 was purchased from Transduction Laboratories. Anti Fas IgM antibody CH11 was from Upstate Biotechnology Inc. and monoclonal anti-PARP antibody was obtained from Oncogene Research Product. Anti-T7 and anti-Flag epitope antibodies were purchased from Merck and Sigma, respectively.

Plasmids

p62 cDNA containing T7 epitope tag at the C-terminus (Joung et al., 1996) was subcloned into the mammalian expression vector pLXSN (Miller and Rosman, 1989) using *Eco*RI and *Xho*I sites for the generation of stable cell lines (p62-T7-pLXSN). For transient expression of tagged p62, a *Nde*I site was introduced at the starting codon of p62 cDNA using polymerase chain reaction amplification and subsequently cloned into pRSET vector in order to fuse to T7 epitope tag at the C-terminus. The cDNA encoding p62-T7 was excised from the above vector and placed into downstream of the sequence encoding Flag tag in pCMV2 eukaryotic expression vector using blunt ended *Nde*I and *Xba*I sites. The resulting construct contained p62 cDNA that was doubly tagged with Flag and T7-epitope at the N- and C-terminis, respectively (Flag-p62-T7-pCMV2).

Site-directed mutagenesis was performed with the QuickChange kit (Stratagen) as recommended by the manufacturer using Flag-p62-T7-pCMV2 clone described above and mutagenesis primers 5'-ATTGAAGTAGATG-CCGCTGTGGAGC-3' and 5'-GCTCCACAGCGGCATC-TACTTCAAT-3'. Positive clones were identified by *Cla*I digestion, and verified by DNA sequencing.

Transfection

For stable transfection, 10^7 Jurkat T cells were transfected with 20 µg of p62-T7-pLXSN by electroporation at 250 V/960 µF using a Gene Pulse apparatus. The transfected cells were plated out 48 h later at limited dilution in media containing 500 µg/ml G418 in 96-well microtiter plates. Single cell originated clones were selected after 2-3 weeks. Empty pLXSN vector was also transfected into Jurkat cells and G-418 resistant cells were isolated and used for control experiments. For transient expression, Jurkat cells were electroporated with Flag-p62-T7-pCMV2, cultured for 40 h, and treated to induce apoptosis.

Cell activation

Exponentially growing Jurkat cells were resuspended at 10^6/ml in PIRM medium containing 10% FCS. Cells were left untreated or incubated with 10 ng/ml of PMA or 1 µM of Ionomycin, respectively, for 12 h. For UV treatment, 4×10^6 cells were resuspended in 0.5 ml phosphate buffered saline (PBS), UVC irradiated (40 J/m^2), and fresh medium was added to the cells. Cells were further incubated for 3 h. Fas-mediated apoptosis in Jurkat cells was accomplished by incubating cells in the presence of CH11 anti-Fas antibody (60 ng/ml) for 4 h at 37℃ otherwise times were indicated. For inhibitor studies, cells were preincubated for 30 min with 50 µM Z-DEVD-FMK before addition of anti-Fas antibody at 100 ng/ml.

Western blot analysis

2×10^6 Cells were lysed in 200 µl of ice cold lysis buffer (20 mM Tris-Cl, pH 8.0, 137 mM NaCl, 1% Triton X-100, 5 mM NaF, 1 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM PMSF) for 20 min on ice. Insoluble materials were recovered by centrifugation at 13,000 g for 15 min and solublized in 50 µl of 10 mM Tris-Cl and 1% SDS for 10 min at room temperature. After addition of 150 µl lysis buffer, samples were sonicated for 20 s with a tip sonicator. For immunoblotting, cell fractions normalized for total protein were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first blocked with 5% milk powder for 1 h, followed by incubation with primary and secondary antibodies, and finally developed with enhanced chemiluminescence (Amersham).

DNA fragmentation analysis

Low molecular weight chromosomal DNA was purified from cells undergoing apoptosis in response to anti-Fas antibody. Briefly, 6×10^5 cells were washed with PBS and lysed in 500 µl 5 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 0.5% Triton X-100. Samples were incubated on ice for 5 min, and insoluble materials were removed by centrifugation. The supernatant was transferred to a new tube, and the nucleic acid fraction was purified by extraction with phenol/chloroform twice followed by ethanol precipitation. The precipitates was dissolved in 15 µl of TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) containing 2 µg/ml RNase A and incubated for 30 min at 37℃. Each sample was separated by 1.8% agarose gel electrophoresis, and DNA was visualized by ethidium bromide staining.

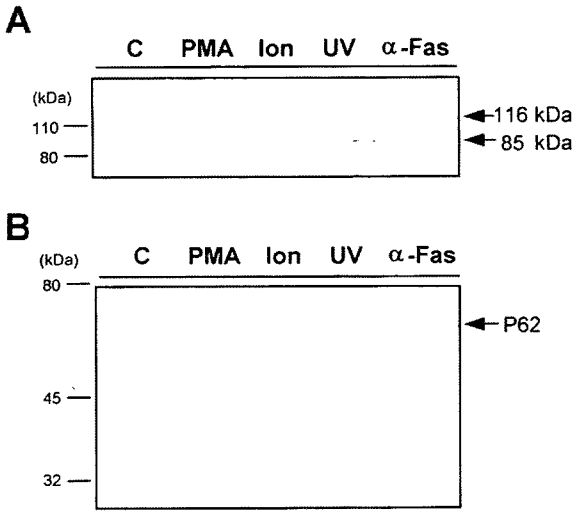

Fig. 1. Disappearance of p62 in response to apoptotic stimuli in Jurkat cells. A, Western blot analysis of whole cell lysates probed with anti-PARP antibody following exposure of Jurkat cells to various reagents. Apoptotic response was determined by PARP cleavage. Antibody used here recognized both full-length and fast migrating apoptotic fragment of PARP, indicated as 116 and 85 kDa, respectively. C; not treated , PMA; PMA (10 ng/ml), Ionomycin (1 μM), UV (40 J/m^2), and α-Fas (60 ng/ml) treated. B, Same cell lysates were analyzed for endogenous p62 protein as indicated by the arrow in immunoblotting using anti-p62 antibody.

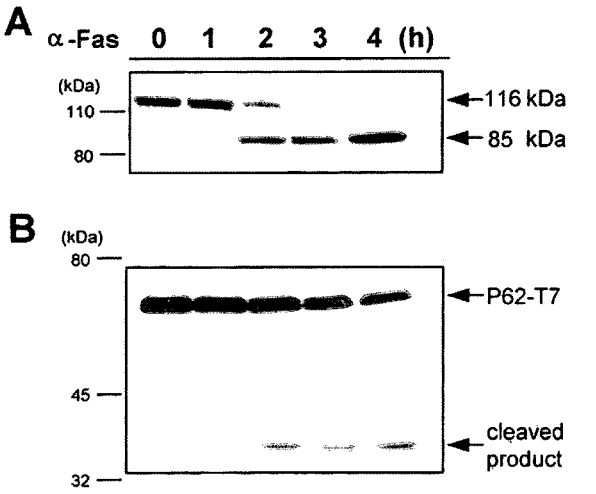

Fig. 2. p62 was proteolytically processed during apoptosis in Jurkat cells. A, p62-T7 expressing Jurkat cells were induced to die by addition of anti-Fas antibody (60 ng/ml) and incubation for the indicated periods of time. Apoptotic progression was determined by increment of 85 kD bands, the cleavage product of PARP, in Western blot analysis using anti-PARP antibody. B, The same cell lysates were analyzed in immunoblotting using anti-T7 antibody. Full-length p62 peptide (shown as 62-T7) was processed to generate a band of about 35 kDa bands indicated as cleaved product.

Results

p62 cleavage during apopotsis in Jurkat cells

To address the potential role of p62 during programmed cell death, lysates of Jurkat cells were examined by Western blot analysis upon induction of apoptosis by Fas receptor-ligation or UV irradiation. Changes in morphology and nuclear condensation indicated that Jurkat cells responded to the apoptotic stimuli (data not shown). The apoptotic response was verified by the cleavage of poly (ADP-ribose) polymerase (PARP) (Fig. 1A). One of the later events observed in cells undergoing apoptosis is activation of caspases, resulting in the proteolytic degradation of cellular substrates such as PARP and lamin (Salvesen and Dixit, 1997). The disappearance of full-length peptide of PARP with molecular weight of 116 kDa was concomitant with the appearance of fast-moving fragment shown as 85 kDa. This cleavage by activated caspase was indicative of apoptotic progression. Interestingly, the amount of p62 decreased in cells undergoing apoptosis. By contrast, in non-apoptotic cells that had been treated with PMA or Ionomycin the amount of p62 remained comparable to control cells (Fig. 1B). In addition, the reduction of p62 was relevant to the extent of remaining uncleaved PARP (shown as UV and Fas in Fig. 1), indicating that the disappearance of p62 is a specific phenomenon related to apoptosis. This observation suggests that p62 itself participates in signal transduction pathways transmitting the death signal.

To investigate the detailed fate of p62, I generated Jurkat cells, in which T7-epitope tagged p62 was ectopically overexpressed. Induction of apoptosis through Fas-ligation resulted in the formation of p62 cleavage product with a molecular weight of about 35 kDa (Fig. 2). The cleaved product shown in Fig. 2B was from the C-terminus since an antibody used in Western blot analysis detected T7-epitope located at the C-terminus of p62. The reason why cleaved product from endogenous p62 was not detected in Jurkat cells might be explained by the low amount of protein since anti-p62 antibody also recognized the C-terminus of p62. This result suggested that p62 itself is a target of proteolytic processing during apoptosis. Interestingly it was observed that intensity of the full-length p62 declined as apoptosis progressed. However, accumulation of the cleaved product peaked at 2 h and subsequently remained up to 4 h after Fas receptor ligation. This indicated that cleaved product could be degraded or subjected to further proteolysis.

The fate of cleaved product of p62 during apoptosis

Since anti-p62 or T7 antibody used in Western blot analysis only recognize full length and C-terminal cleaved product of p62, the fate of N-terminal fragment was unknown. For detailed analysis I constructed a doubly tagged p62 clone with Flag and T7-epitopes at N and C-terminus, respectively. In a preliminary study, the truncation mutant containing the N-terminal 122 amino acids of p62 was barely detectable in detergent-soluble fraction (unpublished result). Therefore cells were transfected with doubly tagged p62 and lysed, subsequently separated into detergent-soluble and

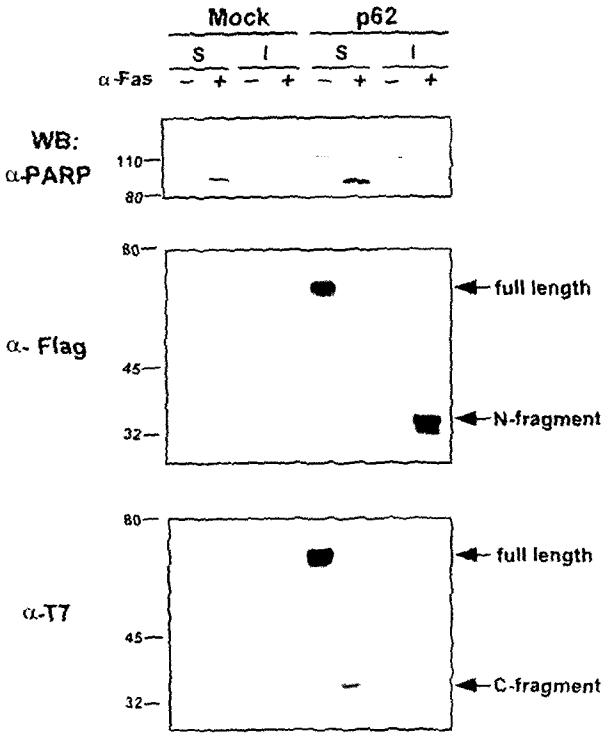

Fig. 3. Cleaved products of p62 showed differential subcellular localizations. Immunoblots showing the N- and C-terminal cleaved products of p62 (shown as N-, and C-fragments) during apoptosis. Jurkat cells were transfected with either expression vector (Mock) or Flag-p62-T7 (p62) and incubated with anti-Fas antibody. Cells were lysed and separated into detergent-soluble (S) and insoluble (I) fractions as described in Materials and Methods. Immunoblot with anti-Flag antibody revealed the full length and N-fragment whereas one with anti-T7 antibody showed the full-length and C-fragment of p62. Apoptotic response of the transfected cells was determined by PARP cleavage (α-PARP).

insoluble fractions to reveal the N-terminal cleaved product. In Western blot analysis probed with anti-Flag antibody the N-terminal cleaved fragment was detectable as about 35 kDa band that was largely detergent-insoluble after Fas receptor-ligation (indicated as N-fragment in Fig. 3). Concomitant with the disappearance of the full-length p62 in the detergent-soluble fraction, the N-terminus of p62 was accumulated in the detergent-insoluble fraction. Interestingly, majority of the full-length and C-terminus fragment of p62 (indicated as C-fragment) was retained in the detergent-soluble fraction. In addition, the intensity of the C-fragment was markedly lower compared to that of the N-fragment, confirming that the C-terminal fragment is further degraded. Taken together, these results indicated that p62 is cleaved into two fragments that differed in subcellular localization in Jurkat cells undergoing apoptosis.

p62 is cleaved by caspase-3 during apoptosis in Jurkat cells

p62 contains a putative ICE/caspase recognition sequence consisted of IEVDIDVE encompassing amino acids 253 to 260 at the hinge region (Fig. 4A). Although

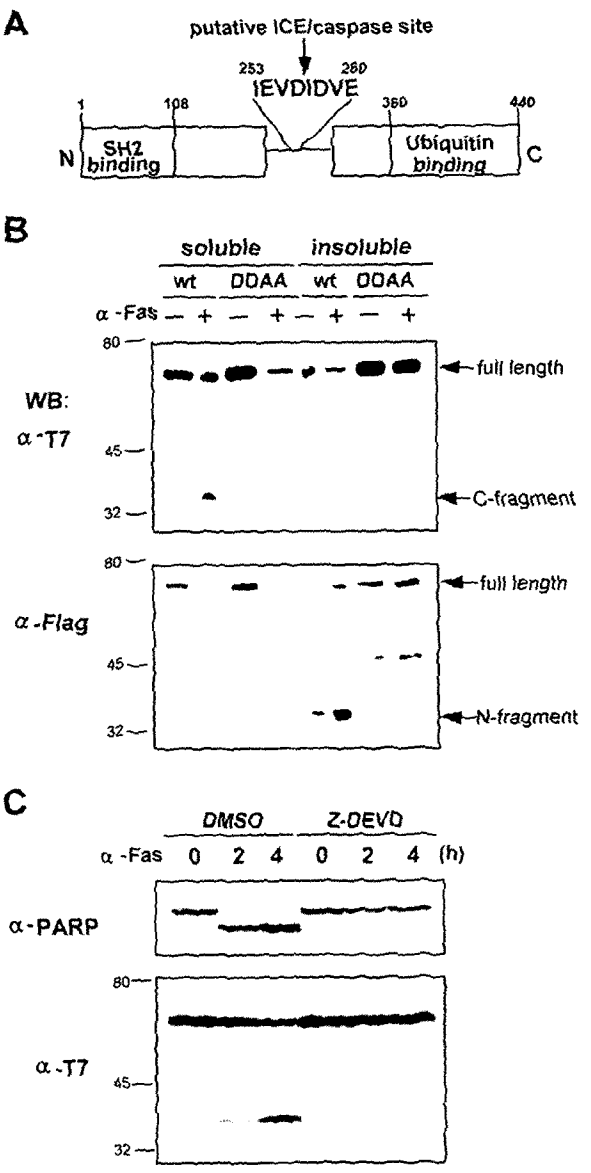

Fig. 4. Caspase-3 is responsible for p62 cleavage during apoptosis in Jurkat cells. A, A schematic representation of p62, which has $p56^{lck}$ SH2 domain and ubiquitin binding domains residing at the N and C-terminus of p62, respectively. These binding domains are separated by a hinge region, where the putative ICE/caspase recognition sequence is located as indicated by the arrow. B, Immunoblots showing that a p62 mutant containing DDAA sequence at amino acid 253 to 256 was not cleaved during apoptosis. Jurkat cells were transfected with either wt (wt) or mutant (DDAA) Flag-p62-T7 and incubated in the presence (+) or absence (-) of anti-Fas antibody. Cells were lysed and separated into detergent-soluble and -insoluble fractions and subjected to Western Blot analysis using anti-T7, and anti-Flag antibodies. C, p62-T7 Jurkat cells were treated with either DMSO or Z-DEVD-FMK for 30 min. prior to addition of anti-Fas antibody and incubated for the indicated periods of time. Cells were lysed in lysis buffer and analyzed. Immunoblots with anti-PARP antibody showed caspase-3 inhibitor completely blocked PARP cleavage. p62 was not cleaved in the presence of Z-DEVE-FMK during apoptosis induced by Fas-ligation.

this sequence is not a perfect match for the caspase-3 cleavage site, DEXD (Nicholson and Thornberry, 1997; Villa et al., 1997), cleavage at this point could generate

two fragments with molecular weights of about 35 and 20 kDa, respectively. The discrepancy in the apparent (35 kDa) and the calculated molecular weight (20 kDa) for the C-fragment might be explained by an unusual migration behavior of the C-fragment during SDS-PAGE or due to migration differences of the molecular weight standard marker. The former could be possible because the full-length p62 with the expected molecular weight of 50 kDa also runs as 62 kDa during SDS-PAGE.

In order to test this possibility the VDID sequence within the potential caspase recognition site of p62 was changed to DDAA. Jurkat cells were transfected with a wild type and a mutated form of doubly tagged p62 and the cell lysates were analyzed. In Western blot analysis using anti-T7 antibody, the C-terminal fragment of wild type p62 was detected in response to Fas-ligation, whereas no cleaved product was found in the detergent-soluble fractions of the DDAA mutant transfected cells (Fig. 4B). As observed above, the wild type p62 was proteolytically processed to generate the N-fragment that was probed with anti-Flag antibody in the detergent-insoluble fraction. In contrast, the mutant p62 peptide remained as full-length and most of them was present in the detergent-insoluble fraction after the induction of apoptosis. It was noted that an additional band with molecular weight of 45 kDa reacting with anti-Flag antibody was present in the lysate from the DDAA mutant transfected cells. It could be shown that this band reflects non-specific cross- reactivity of the antibody or there could be a second cleavage site in p62 peptide. If so, this might explain why the intensity of signals for the C-fragment was considerably weaker than that for the N-fragment as apoptosis prolonged. The results above indicated that p62 is cleaved at the putative ICE/caspase recognition sequence, IEVDIDVE, to generate two fragments.

Further evidence that caspase-3 indeed is responsible for the cleavage of p62 in apoptotic Jurkat cells was given by an analysis using caspase-3 inhibitor. The membrane-permeable irreversible inhibitor Z-DEVD-FMK was added to the p62-T7 expressing cells prior to anti-Fas antibody treatment, and the cell lysates were analyzed. In the presence of Z-DEVD-FMK, formation of the C-fragment as well as PARP cleavage was completely blocked (Fig. 4C), confirming that p62 is primarily targeted by a caspase-3 cleavage during apoptosis in Jurkat cells.

Overexpression of p62 attenuates the Fas ligation induced apoptosis in Jurkat cells

It was assessed whether p62 is involved in the regulation of apoptosis using p62 overexpressing Jurkat cells. Following ligation of Fas receptors, control cells underwent rapid apoptosis characterized by DNA fragmentation within 2 h, whereas the same degree of chromosomal DNA degradation was observed 3 h in the

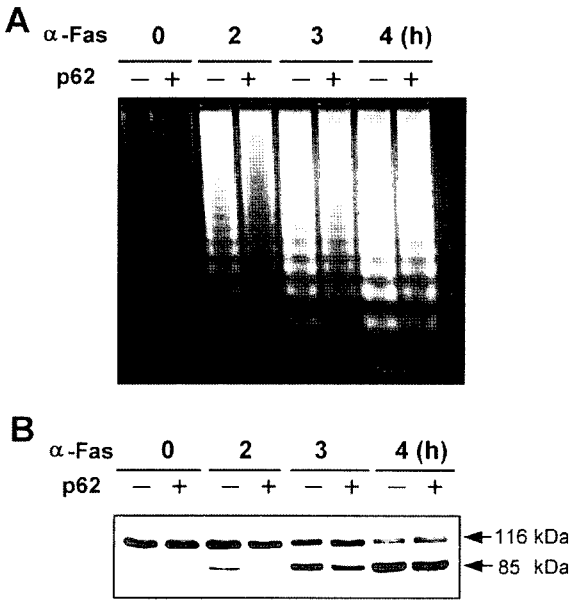

Fig. 5. An inhibitory effect of overexpression of p62 on Fas-mediated apoptosis in Jurkat cells. A, A decrease in DNA fragmentation in p62-T7 expressing Jurkat cells. Control (-) or p62-T7 cells (+) were treated with anti-Fas antibody and incubated for indicated times shown above the figure, and isolated low molecular weight chromosomal DNAs were resolved in agarose gel. B, Attenuation of PARP breakdown in p62-T7 overexpressing Jurkat cells. Control (-) and p62-T7 (+) cells treated same as in (A) were lysed, and the cell lysates were analyzed for PARP by immunoblotting.

p62-T7 expressing Jurkat cells (Fig. 5A). In addition, the onset of PARP cleavage in response to anti-Fas antibody was delayed in the p62 overexpressing cells (Fig. 5B). The inhibitory effect was also observed when apoptosis was induced by UV-irradiation in p62-T7 expressing cells (data not shown), indicating that p62 overexpression resulted in decreased sensitivity of Jurkat cells to apoptotic stimuli.

Discussion

Lymphocyte apoptosis is essential for proper function of the immune system, such as homeostasis of immune cells and elimination of autoreactive lymphocytes. Apoptosis is regulated by a series of biochemical events that lead to a cell death. A common feature of cells undergoing apoptosis is activation of caspases, a family of aspartate-directed proteases (Rowan and Fisher, 1997; Scaffidi et al., 1999). A number of substrates have been reported to be the target of these proteolytic enzymes. Among those, considerable attention has been drawn to caspase-mediated degradation of specific proteins involved in cell growth and survival. Thus, caspase-mediated post-translational processing seems to trigger either activation of pro-apoptotic kinase, such as MEKK-1, or inactivation of anti-apoptotic protein like Akt, and Bcl-2. This process consequently leads to cell death (Widmann et al., 1998).

Here I showed that p62 was target for the proteolytic processing by caspase-3 in apoptotic Jurkat cells. The cleavage generated two fragments that differed in subcellular localization. During apoptosis, the proteins partitioned into the detergent-insoluble fraction containing N-terminal cleaved product, and the detergent-soluble fraction containing C-terminal cleaved product. The cleaved peptides also differed in stability. The N-fragment was stable while the C-fragment was further degraded during apoptosis. The cleavage seemed to occur at ^{253}IEVEIDVE^{260} sequence that resided at the hinge region of the p62 peptide. It is intriguing that this point divides protein into two different domains in terms of associating partners. The N-terminal part is critical for interaction with the p56^{lck} SH2 domain (Joung et al., 1996) and PKCζ (Pulse et al., 1997), whereas the C-terminal 120 amino acids are sufficient for binding to ubiquitin (Vadlamudi et al., 1996). In addition, I have detected the interaction between the truncated p62 peptide containing N-terminal 187 amino acids and 14-3-3τ protein in yeast two hybrid system (unpublished observation). The association between these two proteins was also observed in Jurkat cells. 14-3-3τ is a key player in the cell survival pathway, where it suppresses the action of pro-apoptotic Bad protein through interaction with the phosphorylated Bad in cytoplasm, and subsequently inhibiting the formation of heterodimer between Bad and Bcl-2 at the mitochondrial membrane (Zha et al., 1996). Therefore, p62 has at least two domains separated by caspase-3 cleavage site, one for binding anti-apoptotic proteins such as PKCζ and 14-3-3τ and the other for binding ubiquitin.

Although there is no experimental evidence published, it is possible that after separating from the C-terminus, the N-terminal domain of p62 directs its associating proteins to the detergent-insoluble fraction to form aggregates, in which the N-fragment acts as a scaffold linking other signaling molecules during apoptosis. The previous observation in which atypical PKC was localized into lysosome-targeted endosomes through interaction with p62 supports this hypothesis (Sanchez et al., 1998). In addition, p62 was found as a major constituent in hyaline bodies that resembles inclusions in hepatocarcinoma cells (Stumptner et al., 1999). It is possible that proteins like PKCζ might no longer function as anti-apoptotic kinase by forming aggregates with p62 in detergent-insoluble fraction. This agrees with the observation that overexpression of p62 in Jurkat cells decreased the sensitivity to apoptotic stimuli. Once apoptosis was cued, excess amount of free p62 could saturate caspase activity, so that it delays the process forming aggregates with other proteins involved in cell survival pathway. If this is the case then the precise nature of aggregates and the question whether the N-terminal domain of p62 recruits other signaling proteins into the aggregates during apoptosis is obviously of great importance.

Acknowledgements

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