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Caspase-Dependent Cleavage of c-Abl Contributes to Apoptosis

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The nonreceptor tyrosine kinase c-Abl may contribute to the regulation of apoptosis. c-Abl activity is induced in the nucleus upon DNA damage, and its activation is required for execution of the apoptotic program. Recently, activation of nuclear c-Abl during death receptor-induced apoptosis has been reported; however, the mechanism remains largely obscure. Here we show that c-Abl is cleaved by caspases during tumor necrosis factor- and Fas receptor-induced apoptosis. Cleavage at the very C-terminal region of c-Abl occurs mainly in the cytoplasmic compartment and generates a 120-kDa fragment that lacks the nuclear export signal and the actin-binding region but retains the intact kinase domain, the three nuclear localization signals, and the DNA-binding domain. Upon caspase cleavage, the 120-kDa fragment accumulates in the nucleus. Transient-transfection experiments show that cleavage of c-Abl may affect the efficiency of Fas-induced cell death. These data reveal a novel mechanism by which caspases can recruit c-Abl to the nuclear compartment and to the mammalian apoptotic program.

Besides the general proliferative role of nonreceptor tyrosine kinases, the involvement of these molecules in apoptosis has also been described (4, 41). However, the mechanisms that modulate the proapoptotic activity of nonreceptor tyrosine kinases and the mechanisms that mediate their recruitment to the apoptotic machinery are still largely unknown.

The nonreceptor tyrosine kinase c-Abl has been implicated in apoptotic signaling (41). c-Abl is ubiquitously expressed and highly conserved in evolution (38, 41). Its activity is tightly regulated in the cell, and this is achieved by a set of intramolecular interactions (2, 26). c-Abl resembles Src family kinases and consists of a variable N-terminal unique region of 60 or 80 residues, an SH3 domain, an SH2 domain, and a tyrosine kinase domain. Following the catalytic domain, c-Abl has a large C-terminal region of about 90 kDa. Genetically, this "tail" is crucial for c-Abl function, and mice homozygous for C-terminally truncated c-Abl share most of the phenotypic defects of Abl-null mice (29, 35). This region contains several proline-rich motifs (11), allowing interactions with SH3 and WW domain-containing proteins. Three nuclear localization signals (NLSs) (43) and a nuclear export signal (NES) (32), which allow Abl to shuttle between the cytoplasmic and the nuclear compartments, have been mapped in this region, as have binding sites for F actin and G actin that allow Abl to interact with the cytoskeleton (39). Moreover, the C-terminal region contains a putative DNA-binding domain (22).

Changes in localization and protein-protein interactions result in phosphorylation of different substrates and therefore allow c-Abl to participate in several signal transduction pathways and exert specific functions in response to defined stimuli.

c-Abl activity has been implicated in cell division, cell differentiation, cell adhesion, stress response, and apoptosis (38, 41). Several data support the evidence that cellular localization plays a crucial role in determining the outcome of c-Abl activation in response to different stimuli. Indeed, c-Abl is activated in the cytoplasm upon stimulation of growth factor receptors like platelet-derived growth factor and participates in the transduction of proliferative signals (12, 25). Conversely, in the nucleus, c-Abl is activated during the apoptotic response to DNA damage (13, 49).

The oncogenic form of Abl, BCR-Abl, is a marker of chronic myelogenous leukemia and of a subset of acute lymphocytic leukemias. BCR-Abl expression is characterized by constitutive activity and cytoplasmic localization and results in growth stimulation and protection from several apoptotic stimuli (1, 21, 27). It has been recently shown, however, that when forced into the nucleus, BCR-Abl induces apoptosis (40). This evidence, together with the well-documented activation of nuclear c-Abl upon DNA damage, strongly suggests that c-Abl activation in the nuclei of cells has a proapoptotic function.

Death receptors such as Fas and tumor necrosis factor (TNF) Rp55 are engaged by their respective ligands and trigger cellular apoptosis by activating cysteine proteases of the caspase family. Caspases are responsible for cleavage of protein substrates after an aspartic acid residue. Many caspase substrates are functionally and structurally relevant to the apoptotic program (15, 24, 31).

By using human monocytic leukemia U937 cells, Dan and colleagues (9) have shown that during the apoptotic response to TNF treatment, c-Abl kinase activity is induced in the nucleus in a caspase-dependent fashion, further suggesting a role for c-Abl in apoptosis. However, the molecular requirements for caspase-dependent c-Abl activation have not been investigated.

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Here we report that c-Abl is a novel caspase substrate during apoptosis induced by death receptor stimulation. We identified two major cleavage sites, at D565 and D958. Interestingly, cleavage at D958 removes a small C-terminal piece containing the actin-binding region and the NES. The resulting Abl-M1-D958 cleavage product translocates to the nucleus and sensitizes cells to apoptosis upon Fas stimulation. We propose a model in which cleavage by caspases and its kinase activity are required for c-Abl to contribute to Fas-induced apoptosis. Our data reveal a novel mechanism by which c-Abl participates in the apoptotic program and provide a new link between tyrosine kinases and death receptor-triggered cell death.

MATERIALS AND METHODS

DNA constructs. All of the Abl constructs used in this study were obtained from human c-Abl type 1. pSGT-Abl-wt and pSGTAbl-PP were previously described (2). pSGT-Abl-D565A, pSGT-Abl-D644A, pSGT-Abl-D958A, pSGT-Abl-D958-PP, and pcDNA3-Abl-TM (pcDNA3-Abl-D565A-D644A-D958A) were generated with a Quick Change site-directed mutagenesis kit (Stratagene) by using pSGT-Abl-wt, pSGT-Abl-PP, or pcDNA3-Abl-wt as the template. All mutations were confirmed by sequencing.

pcDNA-Abl-wt and pcDNA-Abl-PP where previously described (10). pcDNA3-Abl-Kin$^-$ (K290R) was obtained by subcloning of pSGT-Abl-Kin$^-$ (K290R) into the vector pcDNA3.

pcDNA3-Abl-M1-D565 and pcDNA3-Abl-M1-D644 were obtained by PCR and subcloning into the vector pcDNA3. Briefly, Abl-M1-D565 and Abl-M1-D644 fragments were generated through amplification with specific oligonucleotides carrying two restriction sites inserted at the 5′ and 3′ ends, respectively (*Bam*HI-*Xho*I for Abl-M1-D565 and *Bam*HI-*Eco*RI for Abl-M1-D644). PCR products were then subcloned into vector pcDNA3 digested with *Bam*HI-*Xho*I for Abl-M1-D565 and with *Bam*HI-*Eco*RI for Abl-M1-D644. pcDNA3-Abl-M1-D958 was generated through amplification of the C-terminal part of c-Abl with specific oligonucleotides, starting before the *Bcl*I site and ending at D958 and inserting the *Eco*RV site at the 3′ end. The PCR product was then subcloned into pcDNA3-Abl-wt digested with *Bcl*I-*Eco*RV.

Cell cultures, transfections, and apoptosis induction. CEM and HuT78 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Abl/Arg$^{-/-}$ immortalized mouse fibroblasts (kindly provided by A. Koleske) were grown in Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum.

HuT78 cells were transfected by electroporation at 240 V (950 μF) with 30 μg of specific DNA and 10 μg of a green fluorescent protein (GFP)-expressing plasmid. Ficoll gradient centrifugation was performed 4 h after transfection to eliminate dead cells and debris. Transfected cells were stimulated with 150 ng of anti-Fas immunoglobulin M monoclonal antibody (CH11; UBI) per ml 24 h after transfection. Specific apoptosis was determined as follows: (% of apoptotic cells with anti-Fas − % of apoptotic cells without anti-Fas)/(100 − % of apoptotic cells without anti-Fas) × 100. The typical baseline level of apoptosis in untreated HuT78 cells transfected with the pcDNA3 vector, as well as with all of the Abl constructs, was around 25% in all experiments. All of the graphs show the mean results of at least three independent experiments.

To obtain stable cell lines, HuT78 cells were transfected with pcDNA3 or pcDNA3-Abl-M1-D958 by electroporation as described above and selected with G418 (Invitrogen) at 1 mg/ml.

HEK 293 cells were transfected by the calcium phosphate precipitation method. Proteins were extracted 40 h after transfection.

In the experiment in which Abl mutants were cotransfected in the presence of caspase 8, cells were incubated in the presence of 40 μM z-Val-Ala-Asp-Fluoromethylketone (zVAD). At 40 h after transfection, the zVAD was removed or not removed from the medium to allow caspase activation. Proteins were extracted 1.5 h after zVAD removal.

Abl/Arg$^{-/-}$ fibroblasts were transfected with Lipofectamine (Invitrogen) in 6-cm-diameter Falcon plates by using 15 μl of Lipofectamine and 5 μg of total DNA in each plate. The Lipofectamine was removed after 5 h. Proteins were extracted 24 h after transfection.

CEM cells were treated with 250 ng of anti-Fas immunoglobulin M monoclonal antibody (CH11; UBI) per ml or 100 ng of human TNF-α (Sigma) per ml for the indicated times. Where indicated, cells were stimulated in the presence of 40 μM zVAD (Biomol), which was added 30 min before stimulation.

Protein extracts, immunoblotting, and immunoprecipitation. Following transfection or stimulation, cells were lysed in IP buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 1 mM orthovanadate, 10 μg of tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK] per ml, 5 μg of *N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK] per ml, 1 μg of leupeptin per ml, 10 μg of soybean trypsin inhibitor per ml, 1 μg of aprotinin per ml) and cell debris were eliminated by centrifugation at 16,000 × *g* at 4°C for 10 min. To separate the nuclear fraction from the cytoplasmic fraction, cells were lysed in hypotonic buffer (10 mM HEPES [pH 7.9], 1 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, and all of the protease and phosphatase inhibitors described above). After 5 min of incubation on ice, nuclei were harvested by centrifugation at 16,000 × *g* at 4°C for 30 s. The cytoplasmic fraction was recovered, and nuclear proteins were extracted from the pellet in nucleus buffer (20 mM HEPES [pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and all of the protease and phosphatase inhibitors described above) for 1 h at 4°C on a rotating wheel. For immunoblotting 50 to 80 μg of protein was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose membrane, and detected with specific antibodies. The following antibodies were used for immunoblotting: rabbit polyclonal anti-Abl K-12 (Santa Cruz), which is directed against the catalytic domain, diluted 1:1,000 in phosphate-buffered saline (PBS)–1% milk–0.1% Tween 20; mouse anti-Abl monoclonal antibody 8E9 (Pharmingen), which is directed against the catalytic domain, diluted 1:250 in PBS–1% milk–0.1% Tween 20; mouse anti-Abl monoclonal antibody Ab-3 (Calbiochem), which is directed against the C terminus of Abl, diluted 1:500 in PBS–1% milk–0.1% Tween 20; mouse anti-caspase 8 monoclonal antibody 5F7 (UBI) diluted 1:1,000 in PBS–0.1% Tween 20–1% milk; mouse antiphosphotyrosine monoclonal antibody 4G10 (UBI) diluted 1:1,500 in PBS–3% bovine serum albumin–0.1% Tween 20.

For immunoprecipitations, protein extracts prepared as described above were incubated for 1 h at 4°C with protein A-Sepharose-conjugated K-12 antibody.

Abl kinase activity assay. Abl proteins were immunoprecipitated from total extracts with anti-Abl antibody K-12 (Santa Cruz) coupled to protein A-Sepharose beads. Beads were washed two times with IP buffer without NaCl and two times with kinase assay buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol). A 20-μl volume of kinase assay mix (1 μg of glutathione *S*-transferase [GST]–Crk121-226, 0.5 μl of [γ-^{32}P]ATP [Amersham], 0.1 mM ATP in kinase assay buffer) was added, and the mixture was incubated at room temperature for 10 and 20 min. The kinase reaction was stopped by adding SDS sample buffer and analyzed by SDS-PAGE. Quantification of the reaction was done by cutting the desired bands from the gel, followed by scintillation counting and phosphorimager analysis. The graph shows the mean results of three independent experiments.

In vitro caspase activity assay. Wild-type Abl (Abl-wt) and the Abl-D565A, Abl-D644A, Abl-D958A proteins were produced in vitro by using the rabbit reticulocyte lysate system for coupled in vitro transcription-translation (TNT; Promega) in the presence or absence of ^{35}S-labeled methionine. The equivalent of 60 ng of purified caspases, generously provided by P. Vandenabeele (37), was incubated with one-fifth of the in vitro translation mixture (5 μl) in a total volume of 20 μl of caspase buffer (50 mM HEPES [pH 7.5], 10 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 10 μg of soybean trypsin inhibitor per ml, 1 μg of aprotinin per ml) for 1 h at 37°C as previously described (23). The reaction products were then separated by SDS-PAGE and analyzed by autoradiography or immunoblotting.

Immunofluorescence analysis. Abl/Arg$^{-/-}$ mouse fibroblasts were plated onto coverslips 16 h before transfection. On the following day, cells were transfected with Lipofectamine (Invitrogen) with 3 μg of pcDNA3-Abl-wt or pcDNA3-Abl-M1-D958. At 24 h after transfection, cells were fixed for 10 min in PBS–4% paraformaldehyde and permeabilized in PBS–0.5% Triton X-100. Abl protein was visualized with monoclonal antibody 8E9 (Pharmingen) diluted to 30 μg/μl in blocking buffer (10% fetal bovine serum, 0.1% Triton X-100 in PBS), followed by fluorescein-conjugated anti-mouse antibody (Bodipy; Molecular Probes) diluted 1:200 in blocking buffer. Actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes) diluted 1:40 in blocking buffer. Nuclei were visualized with Hoechst 33342 (Molecular Probes) diluted 1:20,000 in PBS–0.1% Triton X-100. All antibodies were incubated for 1 h at room temperature.

RESULTS

c-Abl is cleaved during apoptosis. To address the possible role of c-Abl in caspase-mediated apoptotic signaling, we first

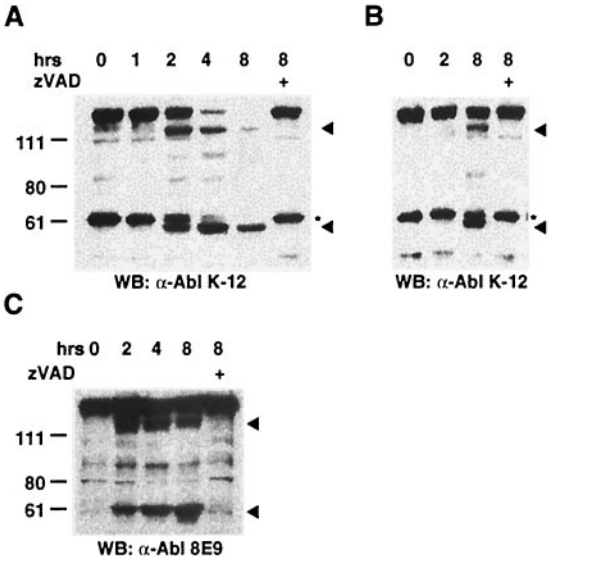

FIG. 1. Caspase-dependent c-Abl cleavage during apoptosis. CEM cells where stimulated to undergo apoptosis by treatment with 250 ng of anti-Fas antibody per ml for 1, 2, 4, or 8 h (A, C) and 100 ng of human TNF-α per ml for 2 or 8 h (B). To inhibit caspase activity, cells were preincubated with 40 μM zVAD and subsequently treated with anti-Fas or TNF-α always in the presence of 40 μM zVAD. Samples (80 μg) of total protein extracts where separated by SDS–7.5% PAGE, blotted to nitrocellulose, and probed with anti-Abl K-12 or anti-Abl 8E9 antibody. The arrowheads indicate two c-Abl cleavage products of about 120 and 60 kDa. The asterisks indicate an unspecific band recognized by the anti-Abl K-12 antibody but not by the anti-Abl 8E9 antibody. This cross-reacting band is also detected by the anti-Abl K-12 antibody in cellular extracts from Abl/Arg$^{-/-}$ fibroblasts, confirming that it is not an Abl product. WB, Western blot. The values on the left are molecular sizes in kilodaltons.

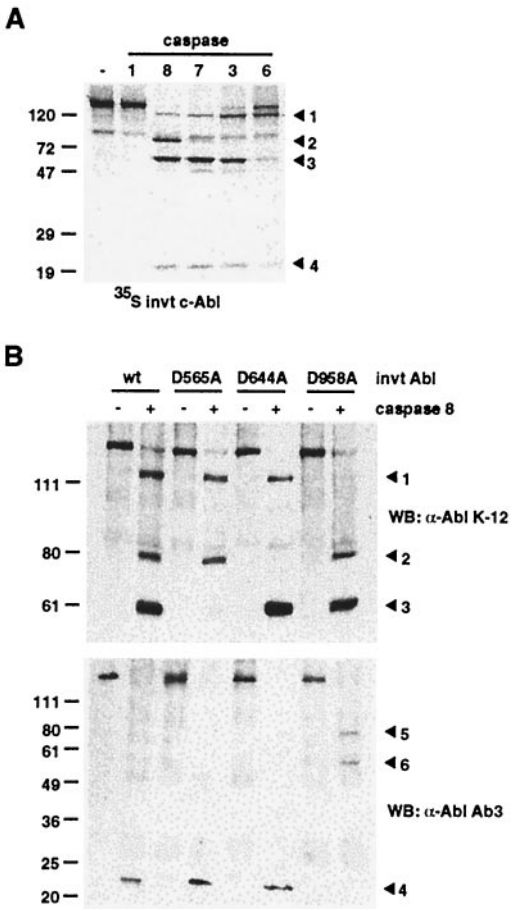

FIG. 2. c-Abl is cleaved by caspase in vitro at three sites. (A) c-Abl was expressed by in vitro translation in rabbit reticulocyte lysate and labeled in the presence of [^{35}S]Met. The in vitro-translated (invt) protein was then processed for in vitro caspase assay in the presence of recombinant purified caspases for 1 h at 37°C. Proteins were then separated by SDS–10% PAGE and revealed by autoradiography. The arrowheads indicate the appearance of four major cleavage products. (B) Abl-wt, Abl-D565A, Abl-D644A, and Abl-D958A were expressed by in vitro translation in rabbit reticulocyte lysate. The in vitro-translated proteins were then incubated in the absence or in the presence of 60 ng of recombinant purified caspase 8 for 1 h at 37°C. Proteins where then separated by SDS–7.5% PAGE (top) or SDS–12% PAGE (bottom), blotted to nitrocellulose, and probed with anti-Abl K-12 (top) or anti-Abl Ab3 (bottom) antibody. Abl caspase cleavage products are indicated by the arrowheads. wt, wild type; WB, Western blot. The values on the left are molecular sizes in kilodaltons.

investigated whether c-Abl could be a substrate for caspases during death receptor-induced apoptosis. Human lymphoid tumor CEM cells were triggered to undergo apoptosis by Fas clustering or TNF exposure, and c-Abl protein was analyzed by Western blotting with the K-12 antibody, which is directed toward the catalytic domain. Figure 1 shows that as early as 2 h after Fas clustering (Fig. 1A) and 8 h after TNF exposure (Fig. 1B), two major Abl fragments of about 120 and 60 kDa accumulated. Pretreatment of cells with the general caspase inhibitor zVAD completely prevented the generation of the cleaved fragments, suggesting involvement of caspases in the process. To further confirm the identity of the two Abl fragments, extracts from CEM cells triggered to undergo apoptosis by Fas clustering were also probed with the 8E9 antibody directed against the catalytic domain of Abl, which identified the accumulation of the 120- and 60-kDa Abl fragments upon caspase activation (Fig. 1C).

c-Abl can be cleaved by caspases at three sites. To investigate whether Abl can be cleaved directly by caspases, in vitro-translated, ^{35}S-labeled Abl was exposed to recombinant purified human caspases in an in vitro cleavage assay. Several caspases, including caspases 3, 6, 7, and 8, but not caspase 1, could cleave Abl (Fig. 2A). Although with different relative efficiencies, four major cleavage products were generated by all of the caspases, giving rise to bands of about 120, 75, 60, and 22 kDa (numbered 1 to 4 in Fig. 2).

On the basis of the size of the in vitro-generated fragments and the known consensus sequences for caspase cleavage, we identified three potential cleavage sites in Abl, occurring after D565, D644, and D958, respectively. We generated three different mutants, each carrying an Asp-Ala substitution within one of the putative cleavage sites. Each mutant form was exposed to purified caspase 8 in vitro, and cleavage products were revealed by the K-12 antibody, which is directed against the catalytic domain of c-Abl (Fig. 2B, top), or the Ab3 antibody, which is directed toward the C-terminal region of c-Abl (Fig. 2B, bottom). Analysis of the proteolytic products revealed that the D565A, D644A, and D958A mutant forms did not allow generation of the 60-, 75-, and 120-kDa fragments,

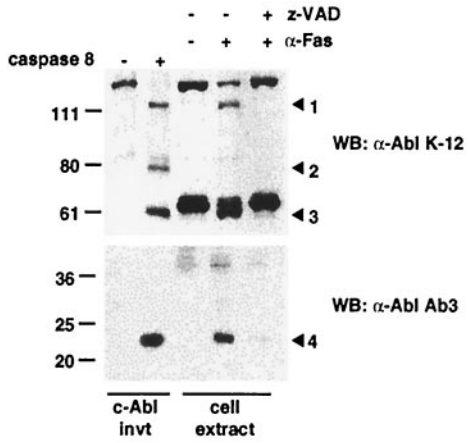

FIG. 3. c-Abl is cleaved in vivo by caspases mainly at two sites. In vitro-translated (invt) c-Abl protein was incubated with or without 60 ng of purified caspase 8 in vitro at 37°C for 1 h as described in the legend to Fig. 2. CEM cells where treated for 4 h with 250 ng of anti-Fas antibody per ml in the absence or in the presence of 40 μM zVAD. In vitro-translated proteins and cell extract proteins were then separated by SDS–7.5% PAGE (top) or SDS–12% PAGE (bottom), blotted to nitrocellulose, and probed with anti-Abl K-12 (top) or anti-Abl Ab3 (bottom) antibody. Abl caspase cleavage products are indicated by the arrowheads. WB, Western blot. The values on the left are molecular sizes in kilodaltons.

respectively, as revealed by the K-12 antibody. Accordingly, the D958 mutant form could not generate the 22-kDa fragment, as revealed by the Ab3 antibody. Importantly, the observation that the Ab3 antibody detected the accumulation of proteolytic intermediates not from the wild type or from the D565A and D644A mutant forms but only from the D958A mutant form (bands 5 and 6 in Fig. 2) suggests that D958 is the predominant cleavage site.

Two cleavage sites are relevant in vivo. We next compared the Abl fragments generated in vitro by caspase 8 exposure with the fragments observed in vivo in CEM cells after Fas cross-linking. As shown in Fig. 3, while bands at 120, 60 (detected by the K-12 antibody, which is directed against the catalytic domain [top]), and 22 (detected by the Ab3 antibody, which is directed against the C-terminal region [bottom]) kDa were detected both in vitro and in vivo, the 75-kDa band could only be detected in vitro. This indicates that cleavage at D644 is not likely to occur in vivo or that the fragment generated is rapidly cleaved at D565. Moreover, the fact that, in vivo, the Ab3 monoclonal antibody detected only the accumulation of the 22-kDa fragment indicates that larger intermediate fragments are rapidly degraded and/or cleaved at D958.

Figure 4 is a schematic representation of c-Abl with all of the caspase cleavage sites marked. All of the in vivo caspase cleavage products are depicted as solid lines, while the cleavage pieces produced only in vitro are shown as dotted lines. Interestingly, the cleavage sites we identified do not alter the catalytic domain. They map to the C-terminal region and cause the loss of important cellular localization signals and protein and DNA-binding sequences. Cleavage at D565 occurs in vivo and in vitro and generates a 60-kDa fragment that retains the SH3 domain, the SH2 domain, and the kinase domain but loses both NLSs and the NES, as well as the DNA-binding and actin-binding regions (fragment 3). This cleavage also generates an 80-kDa C-terminal fragment (fragment 5) that fails to accumulate, probably because of rapid degradation and/or cleavage at D958. Cleavage at D644 was detected only in vitro and mapped just C terminally to the first NLS (fragment 2). The D958 cleavage generates 120- and 22-kDa fragments. The 120-kDa fragment (fragment 1 in Fig. 4) retains the SH3 domain, the SH2 domain, the kinase domain, the DNA-binding region, and the NLSs but loses the actin-binding region and the NES, which are retained in the 22-kDa fragment (fragment 4). Thus, caspase cleavage generates forms of Abl with altered biochemical and biological properties.

Abl cleavage at D958 occurs mainly in the cytoplasm and

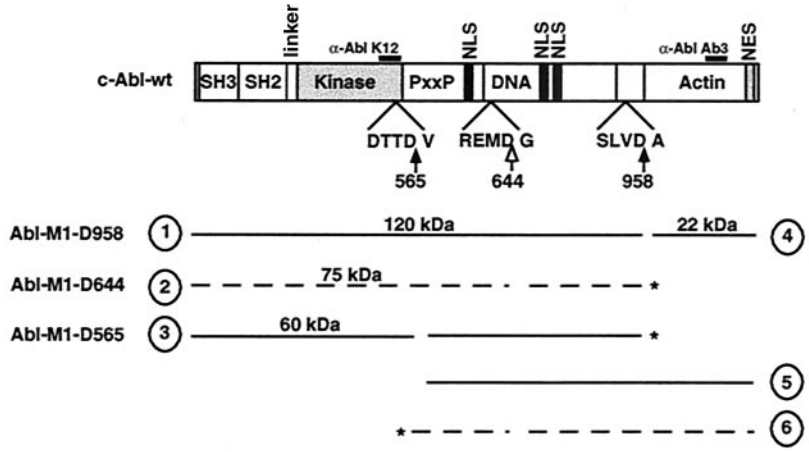

FIG. 4. Schematic representation of c-Abl caspase cleavage products. In the upper part, c-Abl-wt is schematically represented. The functional domains and the anti-Abl antibodies used in this study are depicted. The cleavage sites identified on the human sequence are also indicated by the arrowheads. In the lower part, the Abl cleavage products are shown. Cleavage products generated both in vitro and in vivo are depicted as solid lines, while fragments revealed only in vitro are shown as broken lines. Fragments that retain the catalytic activity are named, respectively, Abl M1-D958, Abl-M1-D644, and Abl M1-D565 and correspond to fragments 1, 2, and 3 in Fig. 2. Fragments containing the portion at the C terminus of the cleavages are numbered 4, 6, and 5, respectively, as in Fig. 2. The central fragments produced by a combination of cleavages, indicated by the asterisks, were not detectable with the antibodies we used.

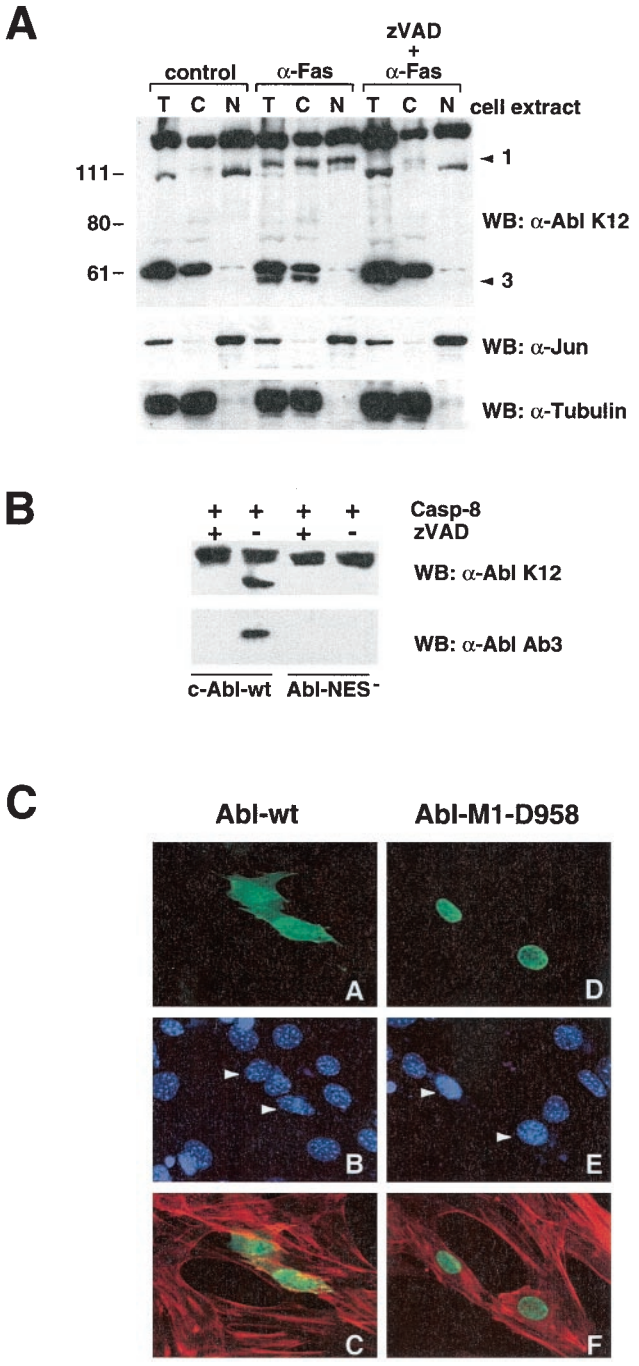

FIG. 5. Caspase cleavage at D958 relocalizes Abl-M1-D958 to the nucleus. (A) CEM cells were incubated with 250 ng of anti-Fas antibody per ml in the presence or absence of 40 μM zVAD. Total (T) extracts and nuclear (N) and cytoplasmic (C) fractions were prepared. Proteins were separated by SDS–7.5% PAGE, transferred to nitrocellulose, and probed with anti-Abl antibody K-12 (top). The purity of the nuclear and cytoplasmic fractions was checked by Western blot (WB) analysis with anti-Jun (middle) and antitubulin (bottom) antibodies, respectively. Arrowheads show Abl cleavage products numbered as in Fig. 4. The values on the left are molecular sizes in kilodaltons. (B) HEK 293 cells were transiently transfected with Abl-wt or Abl-NES$^-$ together with caspase 8 (Casp-8) in the presence of the caspase inhibitor zVAD. To allow caspase activation, zVAD was removed (−) or not removed (+) 1.5 h before cells were lysed and proteins were extracted. Proteins were separated by SDS–7.5 or 12% PAGE, transferred to nitrocellulose, and probed with anti-Abl antibody K-12 (top, where the 120-kDa fragment can be detected) or anti-Abl antibody Ab3 (bottom, where the corresponding C-terminal fragment can be detected), respectively. (C) Subcellular localization of Abl-wt and Abl-M1-D958 by immunofluorescence analysis. Abl/Arg$^{-/-}$ cells were plated onto coverslips 16 h before transfection with Abl-wt or Abl-M1-D958. At 24 h after transfection, cells were fixed and Abl proteins were stained with mouse anti-Abl monoclonal antibody 8E9 (green) as described in Materials and Methods. Nuclei were revealed by Hoechst staining (blue), while the cytoskeleton was highlighted with rhodamine-conjugated phalloidin (red). The arrowheads point to the nuclei of transfected cells.

relocalizes Abl protein to the nucleus. To further analyze the effect of caspase cleavage on the subcellular localization of c-Abl, CEM cells were induced to undergo apoptosis by Fas clustering and cell lysates were prepared after 2 h. Cytoplasmic and nuclear cell extracts were Western blotted with anti-Abl antibody K-12 and with antitubulin or anti-Jun antibodies, respectively, to assess the purity of the fractions. As shown in Fig. 5A, the 120-kDa fragment, corresponding to Abl-M1-D958, accumulates both in the cytoplasm and in the nucleus, while the 60-kDa fragment, corresponding to Abl-M1-D565, accumulates only in the cytoplasm. The 120-kDa fragment lost the NES and therefore the ability to be exported from the nucleus. Its presence in the cytoplasm suggests that, at least in part, cleavage at D958 occurs in the cytoplasm and, once generated, the 120-kDa fragment may relocalize to the nucleus.

To confirm this hypothesis, we took advantage of an Abl construct that carries a point mutation in the NES, Abl-NES$^-$, and therefore accumulates predominantly in the nucleus and we confirmed that c-Abl is cleaved by caspases mainly in the cytoplasmic compartment. Briefly, we have generated a cellular system that mimics the cascade of caspase activation and downstream Fas receptor stimulation. Overexpression of caspase 8 in mammalian cells is sufficient to trigger the cascade and drives the apoptotic program. Therefore, we cotransfected HEK 293 cells with c-Abl-wt, or with Abl-NES$^-$ together with caspase 8 in the presence of the general caspase inhibitor zVAD in the medium. We then removed zVAD from the medium to allow caspase 8 activation, apoptosis progression, and Abl cleavage. While the c-Abl-wt protein is cleaved upon caspase activation, the nuclear Abl-NES$^-$ mutant form is not processed, suggesting that the cleavage occurs preferentially in the cytoplasm and allowing the conclusion that the 120-kDa fragment is mainly generated in the cytoplasm and then relocalized to the nucleus (Fig. 5B).

To further investigate the cellular localization of the 120-kDa fragment, we generated a construct for expression of the Abl-M1-D958 fragment, corresponding to the 120-kDa caspase cleavage product. We expressed the Abl-M1-D958 construct in Abl/Arg$^{-/-}$ fibroblasts, established from mice in which the genes encoding both Abl family members Abl and Arg had been inactivated (16), and revealed its localization by immunofluorescence analysis. Figure 5C shows that, unlike Abl-wt, which is distributed both in the cytoplasm and in the nucleus, the 120-kDa fragment is relocalized exclusively in the nucleus. This is likely to be due to the retention of the NLSs and the loss of the NES. Together, these three approaches suggest that, during the apoptotic process, Abl is mainly cleaved

by cytosolic caspases and that while the 60-kDa fragment is mostly retained in the cytoplasm, the 120-kDa fragment accumulates in the nucleus.

Ability of c-Abl to sensitize cells to Fas-induced apoptosis requires caspase-dependent cleavage of c-Abl, as well as its kinase activity. To investigate the functional role of c-Abl kinase in Fas-induced apoptosis, we used the Fas-sensitive lymphoid cell line HuT78. Upon Fas cross-linking, HuT78 cells showed caspase-dependent cleavage of endogenous c-Abl, resulting in accumulation of the 120- and 60-kDa fragments (data not shown). HuT78 cells were therefore cotransfected with a construct encoding the Abl-wt protein in the presence of a plasmid encoding GFP to allow detection of transfected cells. Overexpression of the entire c-Abl protein, per se, did not induce significant apoptosis in this system (data not shown). By contrast, c-Abl overexpression accelerated by about twofold the rate of Fas-induced apoptosis. Six hours after anti-Fas antibody treatment, in fact, ~25% of the cells transfected with the empty vector showed the apoptotic morphology. Conversely, c-Abl overexpression already resulted in 25% apoptotic cells after 3 h of treatment and produced about 50% apoptosis at 6 h (Fig. 6A).

To assess the requirement for caspase cleavage in the proapoptotic function of c-Abl, we produced a caspase cleavage-resistant mutant form of Abl, Abl-TM, that carries Asp-Ala substitutions at all of the Abl caspase cleavage sites (D565A, D644A, and D958A). These mutations do not interfere in the kinase activity of c-Abl (data not shown). Remarkably, overexpression of the caspase-resistant mutant form of Abl protected transfected cells from Fas-induced apoptosis (Fig. 6A).

To explore the role of Abl kinase activity in Fas-induced apoptosis, HuT78 cells where transiently transfected with Abl-wt or Abl-Kin$^-$ and subsequently incubated in the presence of the agonistic anti-Fas antibody. Interestingly, Abl-Kin$^-$ could not sensitize cells to apoptosis but, conversely, resulted in a delay of cell death (Fig. 6B), similar to what was observed with the caspase-resistant mutant form Abl-TM. Figure 6C shows that, upon transfection, HuT78 cells express all of the Abl mutant forms at the same level. Together, these data strongly suggest that both caspase cleavage and catalytic activity induction are required for the proapoptotic function of c-Abl and for efficient Fas-induced apoptosis. Indeed, an in vitro kinase assay of immunoprecipitated c-Abl protein from Fas-stimulated cells further confirmed that endogenous c-Abl kinase activity is induced during Fas-induced apoptosis (Fig. 6D).

Caspase-dependent cleavage of c-Abl sensitizes cells to apoptosis through accumulation of the Abl-M1-D958 fragment. To further investigate the requirement of caspase-dependent cleavage for the proapoptotic function of c-Abl upon Fas receptor stimulation, we tested the ability of the Abl cleavage products to affect the rate of apoptosis in the same system.

For this purpose, we produced Abl constructs to drive the expression of all of the Abl caspase-dependent cleavage products, Abl-M1-D958, Abl-M1-D644, and Abl-M1-D565. These constructs expressed the corresponding proteins well, as detected upon HuT78 cell transfection and anti-Abl Western blot analysis (Fig. 7A, top).

Interestingly, while Abl-M1-D565 and Abl-M1-D644 did not accelerate the rate of Fas-induced apoptosis, overexpression of Abl-M1-D958 resulted in an increase in apoptosis, as observed with the entire c-Abl-wt protein, suggesting that the proapoptotic effect of c-Abl is mediated by the caspase-generated Abl-M1-D958 fragment (Fig. 7A, bottom). Surprisingly, the Abl-M1-D958 fragment sensitizes cells to Fas-induced apoptosis less efficiently than the complete c-Abl-wt protein. We cannot completely explain these data. However, we can speculate on a contribution to the apoptotic program by the very C-terminal fragment of Abl released upon caspase cleavage at D958.

To further confirm the ability of Abl-M1-D958 to sensitize cells to Fas-induced apoptosis, we established a pool of cells stably transfected with this construct that express very low levels of Abl-M1-D958, even lower than the levels of endogenous c-Abl, and should therefore reproduce the physiological situation more faithfully (Fig. 7B, top). Again, despite the very low levels of expression, Abl-M1-D958 causes a slight but very reliable increase in the rate of Fas-induced apoptosis (Fig. 7B, bottom)

The observation that overexpression of Abl-M1-D958, by itself, does not induce apoptosis (data not shown) yet requires Fas stimulation suggested again that other events, in addition to caspase-dependent cleavage, may be required to switch on the proapoptotic activity of c-Abl. We have shown that c-Abl kinase activity is induced upon Fas stimulation and it is required for the proapoptotic function of c-Abl. We cannot, however, predict whether or how these two events are linked. To assess whether the proteolytic event, per se, drives kinase activation, we analyzed the state of tyrosine phosphorylation of Abl-M1-D958. We have previously shown that tyrosine phosphorylation is a reliable indicator of Abl catalytic activity and that mutation of the two prolines in the linker region (PP) produced strong tyrosine kinase activity (2). Abl-M1-D958 did not show any phosphotyrosine signal upon overexpression in HEK 293 cells, suggesting that cleavage at D958 does not activate the catalytic activity by itself and that the fragment is still regulated (Fig. 7C). However, Abl M1-D958-PP produced a strong phosphotyrosine signal, comparable to that observed with Abl-PP, indicating that Abl M1-D958 is most likely regulated by the same intramolecular mechanisms shown to regulate Abl-wt. These data, together with the observation that Abl-M1-D958 overexpression in HuT78 cells does not induce apoptosis by itself, are in agreement with the idea that, in addition to caspase cleavage, a second, most likely independent, event leading to kinase activation is required for efficient c-Abl proapoptotic signaling. Interestingly, in vitro, Abl-Kin$^-$ and Abl-PP are both as sensitive as c-Abl-wt to caspase cleavage, suggesting that the state of activity of Abl most likely does not affect the susceptibility of the Abl protein to caspase cleavage (Fig. 7D).

DISCUSSION

Several kinases have been identified as caspase substrates during the apoptotic responses. Akt and FAK (focal adhesion kinase), which are normally implicated in survival signaling, are cleaved and inactivated by caspases during apoptosis (19, 42, 45). Many kinases are recruited to the apoptotic program upon caspase cleavage. Indeed, caspase cleavage may result in the loss of a regulatory domain or in a cellular relocalization of the kinase. The final outcome is loss of kinase activity regula-

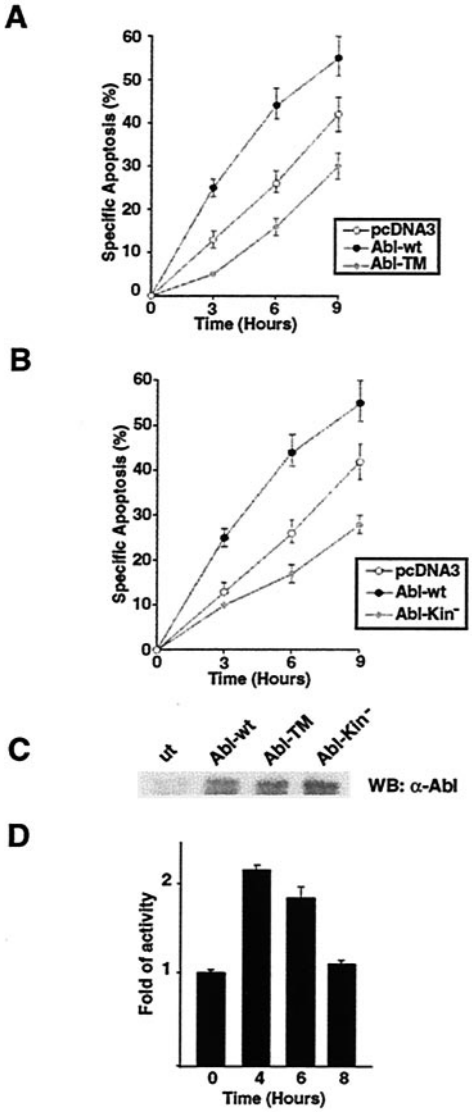

FIG. 6. c-Abl cleavage by caspase and c-Abl kinase activity are required to sensitize cells to apoptosis. (A) HuT78 cells were transfected with vector pcDNA3, with Abl-wt, or with Abl caspase-resistant mutant form Abl-TM, which carries all of the identified cleavage sites carrying the Asp-Ala mutation (D565A, D644A, and D958A), in the presence of a plasmid encoding GFP. At 24 h after transfection, cells were stimulated with 150 ng of anti-Fas antibody per ml. Transfected apoptotic cells were counted at different times, and specific apoptosis was calculated as described in Materials and Methods. The results shown are percentages of specific apoptosis at different times of stimulation. (B) HuT78 cells transfected with pcDNA3, Abl-wt, or Abl-Kin$^-$ and Fas stimulated as described for panel A. Specific apoptosis at different times of treatment was determined as described for panel A. (C) Abl constructs were transiently transfected into HuT78 cells. At 24 h after transfection, total protein extracts were prepared, separated by SDS–7.5% PAGE, blotted onto nitrocellulose, and probed with anti-Abl antibody K-12. ut, untransfected. WB, Western blot. (D) Endogenous c-Abl was extracted and immunoprecipitated with anti-Abl antibody K-12 from HuT78 cells after Fas treatment for different lengths of time. The activity of immunoprecipitated Abl was measured by in vitro kinase assay with the GST–c-Crk protein as the substrate in the presence of [γ-^{32}P]ATP. Bands corresponding to the GST–c-Crk protein were excised, and incorporated radioactivity was measured by scintillation counting. The endogenous Abl activity after Fas stimulation for different lengths of time was normalized for the amount of immunoprecipitated Abl and is shown as fold activity.

tion and the generation of a constitutively active form of the enzyme. This mechanism has been reported for many serine-threonine kinases, like MEKK1 (6, 44), PAK2 (5, 28), and Mst1 (14, 36), and also for some nonreceptor tyrosine kinases, like Fyn and Lyn (20), which belong to the Src family, and Etk (47), which belongs to the Btk family.

We present evidence here that shows, for the first time, that c-Abl is directly cleaved by caspases during apoptosis. c-Abl cleavage by caspases occurs quite early during Fas-induced apoptotic signaling. This observation suggested that c-Abl cleavage may be involved in apoptotic signal progression. Indeed, we have shown that a caspase-resistant mutant form of Abl that carries Asp-Ala substitutions at relevant cleavage sites protects cells from Fas-induced apoptosis.

Abl cleavage generates the Abl-M1-D958 fragment that is ultimately responsible for the proapoptotic effect. The observations that overexpression of Abl-M1-D958, per se, does not induce cell death and that, similar to Abl-wt, it requires Fas stimulation to exert its proapoptotic effect, suggest that caspase cleavage is only one step of the mechanism that switches on the proapoptotic function of c-Abl. According to a previous report by Dan and colleagues (9), our observations that c-Abl kinase activity is induced upon Fas stimulation and that Abl-Kin$^-$ cannot sensitize cells to apoptosis indicate that the second step is induction of tyrosine kinase activity. In agreement with this, BCR-Abl can also be proapoptotic if its tyrosine kinase activity is switched on in the nucleus (40). During Fas-induced apoptosis, c-Abl tyrosine kinase activity induction depends on Fas stimulation but it is most likely not directly caused by proteolytic processing because caspase cleavage at D958, per se, does not affect Abl catalytic activity. This is in agreement with a recent study that shows that the entire C-terminal region of c-Abl is dispensable for the regulation of catalytic activity in vitro (26). The molecular mechanism linking caspase cleavage and c-Abl tyrosine kinase activation needs to be further investigated. The kinase activity of c-Abl in the nucleus is positively regulated during the G_1-S-phase transition by release of the inhibitory interaction with Rb (43). c-Abl activity can be induced by ATM kinase upon DNA damage only in S-phase cells (3, 18, 30), suggesting that release of the Rb interaction is also required for c-Abl activation in this context. Interestingly, not only is Rb cleaved by caspases during apoptosis (33, 34) but, more importantly, fibroblasts from mice expressing caspase-resistant Rb are protected from TNF-induced apoptosis (7). Moreover c-Abl cannot be activated by TNF in mice expressing caspase-resistant Rb (T.-T. Chen and J. Y. J. Wang, unpublished results). Therefore, cleavage of Rb by caspases during apoptosis can provide an intriguing mechanism by which to release c-Abl from the inhibitory interaction with Rb in the nucleus, allowing its further activation.

We are aware of the presence of other c-Abl caspase cleavage products. In vivo, we can follow the production and accumulation in the cytoplasm of an Abl-M1-D565 fragment. This fragment loses the NLSs and the NES and does not relocalize to and accumulate in the nucleus (data not shown). This may explain its failure to sensitize cells to Fas-induced apoptosis. We also mapped an extra caspase cleavage site to D644 in vitro but always failed to detect this fragment in vivo. Nevertheless, we also tested the activity of Abl-M1-D644 in Fas-induced apoptosis and again this fragment did not accelerate the rate of

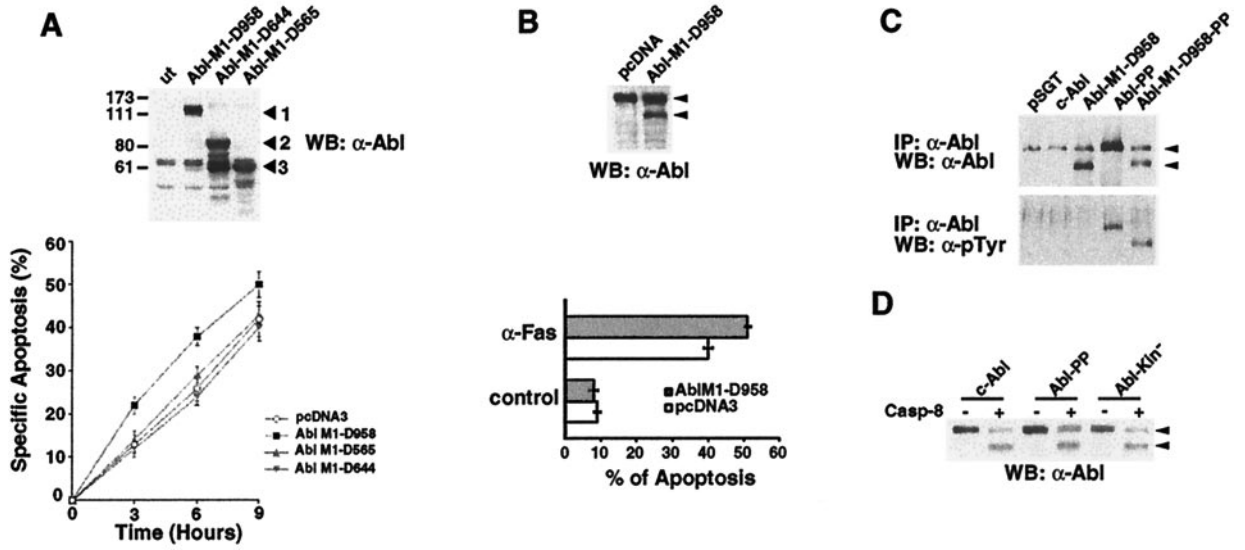

FIG. 7. Abl-M1-D958 mediates the proapoptotic function of c-Abl. (A, top) Abl constructs were transiently transfected in HuT78 cells. At 24 h after transfection, total protein extracts were prepared, separated by SDS–7.5% PAGE, blotted to nitrocellulose, and probed with anti-Abl antibody K-12. Arrowheads indicate the overexpressed Abl fragments. ut, untransfected. The values on the left are molecular sizes in kilodaltons. (A, bottom) HuT78 cells were transfected with several Abl constructs and Fas stimulated as described in the legend to Fig. 6A. Specific apoptosis at different time of treatment was determined as for Fig. 6A. The values on the left are molecular sizes in kilodaltons. WB, Western blot. (B) A pool of HuT78 cells stably transfected with the empty vector (white bars) or with Abl-M1-D958 (grey bars) were treated with 150 ng of anti-Fas antibody per ml. Apoptosis was measured 4 h after stimulation by PI staining and fluorescence-activated cell sorter analysis. The top shows a Western blot analysis of total protein extracts with anti-Abl antibody K-12. The arrowheads indicate endogenous c-Abl and the Abl-M1-D958 transfected construct. The graph at the bottom shows the mean of three independent experiments. (C) HEK 293 cells were transiently transfected with several Abl constructs. Total protein extracts were immunoprecipitated (IP) with anti-Abl antibody K-12. Immunoprecipitated proteins were separated by SDS–7.5% PAGE, blotted onto nitrocellulose, and probed with anti-Abl antibody K-12 (top) or with antiphosphotyrosine antibody (bottom). The arrowheads indicate full-length Abl and the Abl-M1-D958 construct. (D) Abl-wt, Abl-PP, and Abl-Kin$^-$ were expressed by in vitro translation in rabbit reticulocyte lysate. The in vitro-translated proteins were then incubated in the absence or in the presence of recombinant purified caspase 8 (Casp-8) for 2 h at 37°C. Proteins where then separated by SDS–7.5% PAGE, blotted onto nitrocellulose, and probed with anti-Abl antibody K-12. The arrowheads indicate full-length Abl and the Abl-M1-D958 fragment.

cell death. Cleavage at D644 produces a fragment that retains only one of the three NLSs and again lacks the NES. This fragment can accumulate in the nucleus to a lesser extent than Abl-M1-D958 (data not shown). This may partially explain its failure to induce cell death. However, we cannot rule out the possibility that regions between D644 and D958, including the DNA-binding region, are required for the proapoptotic function.

Abl cleavage at D958 causes loss of the last 191 residues. G-actin- and an F-actin-binding regions have been mapped to the C-terminal region (39). Moreover, the very last amino acid residues contain the NES (32). Finally, in the first 40 amino acid residues of the A959-stop fragment, several PXXP motifs that could mediate interactions with SH3-containing proteins are present.

It has been recently proposed that c-Abl kinase activity may be inhibited by filamentous actin (46). Moreover, the cytoskeletal protein PSTPIP1 has been identified as a new c-Abl interactor and substrate that mediates c-Abl interaction with the cytoskeleton and with the PEST-type protein tyrosine phosphatase PTP, which can dephosphorylate and negatively regulates c-Abl activity (8). These data suggest the possibility that c-Abl–cytoskeleton association can be a mechanism by which to modulate c-Abl catalytic activity. Therefore, it is possible that caspase cleavage at D958, releasing the interaction with actin, provides a mechanism for c-Abl activation during Fas-induced apoptosis. Moreover, the 191-amino-acid C-terminal piece released by caspase cleavage may contribute to the mechanism by which Abl sensitizes cells to Fas-induced apoptosis by competing with the entire Abl protein for actin binding. This may partially explain the ability of the entire c-Abl protein to sensitize cells to Fas-induced apoptosis better than the Abl-M1-D958 fragment. In this light, we can also speculate that c-Abl caspase cleavage may cause disruption of the c-Abl–actin interaction, thereby contributing to the cytoskeleton rearrangements that occur during apoptosis.

Interestingly, the Abl-M1-D958 fragment retains three NLSs but loses the NES. We have shown that overexpression of this construct results in Abl-M1-D958 protein accumulation in the nucleus. Cellular fractionation of Fas-induced apoptotic cells and the use of Abl localization mutant forms showed that c-Abl cleavage by caspases may occur in the cytoplasm and that the Abl-M1-D958 fragment may be subsequently translocated to and trapped in the nucleus.

We propose a model in which c-Abl cleavage during Fas-induced apoptosis generates a truncated Abl protein that lacks the NES and accumulates in the nucleus, where c-Abl exerts its proapoptotic function. To our knowledge, this may be the first example of a caspase-dependent tyrosine kinase protein translocation from the cytoplasmic to the nuclear compartment. Interestingly, c-Abl function is clearly dependent on subcellular localization and its nuclear accumulation upon Fas-induced

caspase cleavage is in good agreement with the reports that associate nuclear c-Abl activation with apoptosis induction (38, 41).

We detected c-Abl cleavage by caspases not only in Fas-induced apoptosis but also in TNF-induced and DNA damage-dependent cell death (data not shown). This is not surprising, considering that caspases are widely activated in the apoptotic response. Therefore, our studies may underscore a general mechanism for the recruitment of c-Abl protein to the nuclear compartment and to the apoptotic machinery. However, definition of the physiological role in vivo of c-Abl caspase cleavage in apoptosis requires further investigation. We have provided evidence that c-Abl enhances Fas-mediated killing. Nevertheless, CD40-activated lymphocytes prepared from fetal livers of $Abl^{-/-}$ and $Abl^{+/-}$ mice are as sensitive as their Abl-wt littermates to Fas-induced apoptosis, suggesting that c-Abl is not rate limiting for Fas-induced apoptosis in these cells (S. Cho, personal communication). However, since CD40-activated fetal liver cells show a very low level of apoptosis in response to Fas treatment, further experiments with other Fas-sensitive cells, like thymocytes and hepatocytes, which are frequently used to assess the role of apoptotic proteins in Fas signaling in knockout mouse studies (17, 48, 50), are necessary to definitively clarify the role of c-Abl in Fas-induced apoptosis and in other apoptotic signaling.

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