

Requirement of caspase-mediated cleavage of c-Abl during stress-induced apoptosis

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Abstract

c-Abl protein tyrosine kinase plays an important role in cell cycle control and apoptosis. Furthermore, induction of apoptosis correlates with the activation of c-Abl. Here, we demonstrate the cleavage of c-Abl by caspases during apoptosis. Caspases separate c-Abl into functional domains including a Src-kinase, a fragment containing nuclear import sequences, a fragment with an actin-binding domain and nuclear export sequence. Caspase cleavage increases the kinase activity of c-Abl as demonstrated by *in vitro* kinase assays as well as by auto- and substrate phosphorylation. Cells in which c-Abl expression was knocked down by RNA interference resisted cisplatin- but not TNF α -induced apoptosis. A similar selective resistance against cisplatin-induced apoptosis was observed when cleavage resistant c-Abl was overexpressed in treated cells. Our data suggest the selective requirement of c-Abl cleavage by caspases for stress-induced, but not for TNF α -induced apoptosis.

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Abbreviations: AA, amino acids; SH3, Src homologue region 3; SH2, Src homologue region 2; RNAi, RNA interference; siRNA, small interfering RNA

Introduction

c-Abl is an ubiquitously expressed nonreceptor protein tyrosine kinase, which localizes to the cytoplasm and the nucleus.¹ The N-terminus of c-Abl consists of a Src kinase including Src-homologue regions 2 and 3 (SH2 and SH3). Unlike other Src-kinases, c-Abl has a large C-terminal region that contains nuclear localization motifs,² nuclear export sequences,¹ a DNA binding motif³ and an actin-binding domain at the extreme C-terminus.⁴ Moreover, a large number of factors have been identified which interact with different regions of c-Abl.⁵

Two transforming homologues of c-Abl exist, v-Abl, the transforming gene of the Abelson murine leukemia virus⁶ and BCR/Abl, a fusion gene formed by the chromosomal translocation of c-Abl to the BCR locus.⁷ Expression of the constitutively active tyrosine kinase BCR/Abl has been shown to be associated with chronic myelogenous leukemia's (CMLs). A major breakthrough in CML therapy was the use of an Abl-specific kinase inhibitor STI-571 (Gleevec) to inhibit the growth of BCR/Abl-positive cells *in vitro* and *in vivo*.⁷

c-Abl overexpression alone is not sufficient to transform NIH-3T3 cells.⁸ In contrast, activation of c-Abl leads to cell cycle arrest at the G1/S transition⁹ and apoptosis.¹⁰ Active c-Abl, but not a kinase-deficient mutant form of c-Abl-induced apoptosis suggesting a downstream signaling cascade is involved in c-Abl-induced apoptosis.¹⁰ Consistent with the finding that kinase activity is required for apoptotic signaling, treatments which initiate apoptosis like DNA-damaging agents, TNF α and γ -irradiation have been shown to induce c-Abl kinase activity.^{11,12} Furthermore, cells overexpressing a dominant-negative c-Abl, or Abl $-/-$ cells are partially resistant to DNA-damage-induced apoptosis.¹³

The mechanism by which apoptosis-inducing agents activate c-Abl is not well understood. Two general pathways can be envisaged by which apoptotic stimuli are received and processed by the cell. The so-called death receptors belonging to the TNF receptor family¹⁴ have been identified which activate caspases, a class of cysteine proteases, upon ligation.¹⁵ The second general apoptosis pathway activated by a variety of unrelated stimuli involve mitochondria.¹⁶ Mitochondria respond to apoptotic signals with the release of caspase activating factors from the intermembrane space into the cytosol.¹⁷ Caspases cleave cellular substrates and thereby modify their functions, which as a net effect, results in the complex phenotype of apoptosis.¹⁸ One of the mechanisms of kinase activation in apoptotic cells depends on the cleavage by caspases which separate inhibitory domains from the catalytic domains.^{19,20}

Although caspases have been implicated in the activation of c-Abl in apoptotic cells, the mechanism has not yet been defined. In this study, we describe the cleavage of c-Abl by caspases as a major mechanism of activation in apoptotic cells. Caspases cleave c-Abl at three positions resulting in the separation of the intact Src-homologue kinase, the nuclear import sequences and the actin-binding domain. Furthermore, we show by the use of siRNA-induced knock down of c-Abl and dominant-negative approaches that caspase cleavage of c-Abl is required for DNA-damage-induced, but not for TNF α -induced, apoptosis.

Results

Cleavage of c-Abl during apoptosis

Post-translational modifications play an important role in transducing apoptotic signals. We have performed proteome

analyses of apoptotic cells and identified numerous modified proteins, in most cases, caspase substrates.²¹ In order to investigate low-abundance proteins, which are not detected by silver staining, two-dimensional electrophoresis (2-DE) of Jurkat cells induced to apoptosis with anti-Fas antibody was performed and a number of selected proteins were detected by immunoblot analysis. One of the modified proteins identified by this assay was the c-Abl kinase. In apoptotic cells, a 28 kDa spot was identified using an antibody directed against the C-terminal part of c-Abl. This 28 kDa spot was present in very low amounts in immunoblots of nonapoptotic cells (Figure 1a). The spot of the full-size kinase was only detected in the outermost left side of the 2-DE blots indicating that the intact c-Abl was not resolved in the first dimension (not shown). c-Abl cleavage was therefore confirmed in TNF α -treated, apoptotic U937 cells using an antibody against the c-Abl kinase domain. As shown in Figure 1b, TNF α -induced a sequential cleavage of c-Abl. The first cleavage product of approximately 120 kDa appeared after 2 h followed by the second cleavage product of approximately 60 kDa after 4 h (Figure 1b). At 8 h after induction of apoptosis only the 60 kDa fragment was detectable. Using an antibody directed against the C-terminus of c-Abl, we could detect a cleavage product of about 28 kDa corresponding to the size of the spot identified in immunoblots of 2-DE gels (not shown). These results suggested that c-Abl is first cleaved at the C-terminus to release the 28 kDa fragment followed by the generation of a 60 kDa fragment.

Proteolytic cleavage of c-Abl could also be detected when apoptosis was induced by cisplatin or etoposide (Figure 4c). Furthermore, using an antibody directed against the kinase domain of c-Abl, similar results were obtained in anti-CD95 (Fas/Apo-1)-treated SKW6.4 cells (Figure 1c) and Jurkat cells (not shown) as well as in TNF α -induced HeLa cells (not shown). Antibody directed against the C-terminus of c-Abl (Abi-3) showed a slightly different pattern of cleavage in mouse NIH3T3 cells (Figure 1d). Unlike in human cells, an 85 kDa fragment was detected with this antibody probably due to the lack of a cleavage site in the C-terminus of murine c-Abl.

In vitro cleavage of c-Abl by caspases

When cells were preincubated with the caspase-inhibitor z-VAD-fmk prior to the induction of apoptosis, c-Abl cleavage products were not detected at 8 h suggesting that caspases cleave c-Abl (Figure 1b–d). To further investigate the possibility that caspases are responsible for c-Abl cleavage, *in vitro* translated c-Abl was incubated with the lysate of apoptotic Jurkat cells, which contains active caspases,¹⁹ and we analyzed for c-Abl cleavage. The c-Abl cleavage products observed in this *in vitro* assay resembled the fragments detected previously in apoptotic cells (Figure 2). However, in addition to the expected 120, 60 and 28 kDa bands, a 90 kDa fragment was also detected (Figure 2). As seen in apoptotic cells, z-VAD-fmk prevented the fragmentation of c-Abl, indicating that caspases are responsible in this process.

To determine which caspases cleave c-Abl, *in vitro*-translated c-Abl was incubated with purified caspase-3, -8 (Figure 2) and -10 (not shown). Caspase-3 cleaved c-Abl in a z-VAD-fmk-sensitive manner into the same fragments as in

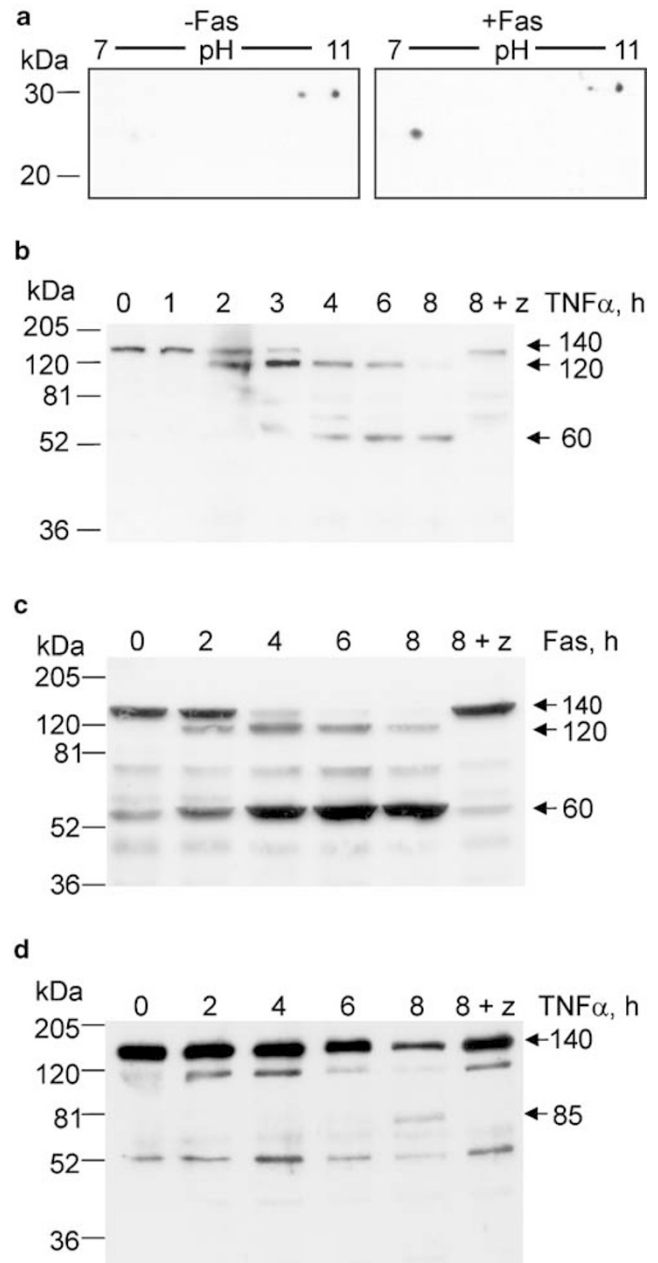


Figure 1 c-Abl is cleaved during apoptosis. (a) Jurkat cells were treated for 6 h with 150 ng/ml anti-Fas antibody (clone CH11, Immunotech) and 1 μ g/ml cycloheximide (apoptotic) or cycloheximide alone (nonapoptotic). Protein lysates were subjected to two-dimensional gel electrophoresis, transferred to PVDF membranes and c-Abl was detected using antibodies directed against the C-terminus (c-Abl C-19). The two spots at 30 kDa correspond to nonspecific binding by the secondary antibody (not shown). (b)–(d) Human U937 cells (B) and mouse NIH-3T3 cells (D) were treated with 50 ng/ml TNF α and 1 μ g/ml cycloheximide, SKW 6.4 cells (C) with 150 ng/ml anti-Fas antibody. After different time points, lysates were prepared, separated by SDS-PAGE, and analyzed by immunoblotting with antibodies directed against the kinase domain (B,C) (8e9) or the C-terminus (Abi-3) of c-Abl. Z=z-VAD-fmk

apoptotic Jurkat cells. Furthermore, caspase-8 and -10 cleaved c-Abl into similar fragments as caspase-3 (Figure 2). Taken together these data show that caspases cleave c-Abl during apoptosis.

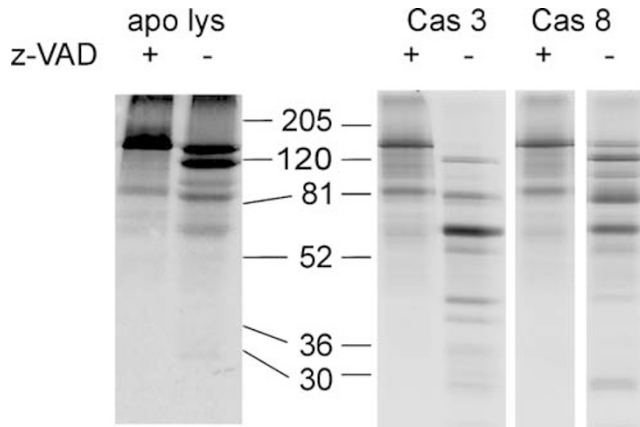


Figure 2 Cleavage of *in vitro* translated c-Abl by apoptotic lysate and caspase-3 and -8. *In vitro* translated c-Abl was treated with apoptotic Jurkat lysates (apo lys) or recombinant caspase-3 or -8 (Cas 3, Cas 8) in the presence or absence of the caspase inhibitor z-VAD-fmk (z-VAD) for 1 h, separated by SDS-PAGE and analyzed by autoradiography

Identification of caspase cleavage sites in c-Abl

Since c-Abl with a molecular mass of 140 kDa is cleaved into a 28 and a 60 kDa fragment, we expected to identify at least two cleavage sites for caspases. Caspases cleave their substrates after aspartates and therefore a putative cleavage site for the generation of the 28 kDa C-terminal fragment was aspartate 957. Cleavage of *in vitro*-translated c-Abl mutated at aspartate 957 to asparagine (c-Abl (D957N)) by caspase-3 generated no 120 and 28 kDa fragments, whereas the 60 kDa fragment was still visible (Figure 3a) suggesting aspartate 957 as one of the caspase cleavage sites in c-Abl. In order to simplify the characterization of the other cleavage sites a deletion mutant of c-Abl, which lacked the amino acids 958–1146 (c-Abl 1–957), was used for further analyses. Since an antibody directed against the kinase domain of c-Abl detected a 60 kDa fragment in lysates of apoptotic cells (Figure 1b–c), aspartates in the kinase fragment which upon cleavage would generate a 60 kDa fragment were mutated. Neither c-Abl (D561N) nor c-Abl (D564N) were cleaved to a 60 kDa fragment by caspases *in vitro* suggesting that both aspartates are necessary for cleavage at $D_{561}TTD_{564}$ (Figure 3b). Thus, the 60 kDa cleavage product of c-Abl exactly represents the N-terminal Src-homologue part of c-Abl (Figure 3e). Since c-Abl 1–957(D561N) and c-Abl 1–957(D564N) were still cleaved by caspases (Figure 3b), we further analyzed c-Abl 1–957 by mutational analyses and thereby identified aspartate D673 as the third caspase cleavage site in c-Abl (Figure 3c). Consistently, the triple mutant c-Abl (D564N, D673N, D957N) resisted cleavage suggesting that all caspase cleavage sites in c-Abl had been identified (Figure 3d).

The presence of the caspase cleavage sites was confirmed in Abl proteins deposited in the public databases. Interestingly, the oncogenic v-Abl p90 gene (Gene Bank accession number P00521) carried a deletion exactly after position 560, suggesting that the absence of the caspase cleavage site $D_{561}TTD_{564}$ may be important for the function of transforming v-Abl p90 (Figure 3f).

Activation of c-Abl by caspase cleavage

The finding that c-Abl is processed by caspases during apoptosis suggested that cleavage modifies the activity of c-Abl during apoptosis. To investigate whether c-Abl is activated by cleavage, U937 cells were induced to undergo apoptosis by $TNF\alpha$ /cycloheximide or etoposide treatment. Using immunoprecipitated c-Abl in *in vitro* kinase assays with the c-Abl substrate Crkl, the appearance of the cleavage products of c-Abl correlated with an increase in the kinase activity of c-Abl (Figure 4a), indicating that caspase cleavage activates c-Abl. The activation of c-Abl correlated with the activation of caspase-3 and was inhibited by the broad range caspase inhibitor z-VAD-fmk demonstrating the importance of active caspases for c-Abl kinase activation (Figure 4b). Similarly, apoptosis induced by etoposide also resulted in cleavage and activation of c-Abl (Figure 4c). Consistent with this result, and as previously demonstrated for $TNF\alpha$, activation of c-Abl by etoposide could be inhibited by the caspase inhibitor z-VAD-fmk suggesting active caspases, rather than the pathway of caspase activation are the cause for c-Abl activation. To investigate whether the Src-like kinase fragment obtained after caspase cleavage is sufficient for Crkl phosphorylation, a recombinant kinase fragment covering amino acids 1–511 was constructed and purified. c-Abl 1–511 was fully active in phosphorylating Crkl *in vitro* (Figure 4d). When immunoprecipitated c-Abl from apoptotic U937 cells was subjected to an *in vitro* kinase reaction in the absence of any substrate, the 120 and the 60 kDa band appeared (Figure 4a). We consistently observed two phosphorylated bands of the 60 kDa fragment, probably due to differential phosphorylation (Figure 4a). As previously observed in *in vitro* kinase reaction with recombinant c-Abl 1–511 (Figure 4d) immunoprecipitated c-Abl fragments were not phosphorylated in the presence of Crkl, suggesting that the substrate phosphorylation is the preferred reaction (Figure 4a). Interestingly, an unknown substrate of 45 kDa co-precipitated with c-Abl from apoptotic U937 cells at later time points (Figure 4a). This substrate, however, was not phosphorylated in the presence of Crkl (Figure 4a).

It has been reported that c-Abl is activated as early as 15 min after induction of DNA damage or cellular stress.²² To test whether this early activation of c-Abl occurs upon $TNF\alpha$ treatment, a kinetics of $TNF\alpha$ -induced c-Abl activation including early time points was performed. Activation of c-Abl coincided with the appearance of cleavage products, which occur as early as 120 min after exposure to $TNF\alpha$ (Figure 4b).

These results suggest a new mechanism for the regulation of c-Abl activity – the caspase-dependent cleavage during apoptosis.

Requirement of c-Abl cleavage for apoptotic signaling

To determine whether c-Abl is required for apoptotic signaling in HeLa cells, we suppressed the expression of c-Abl prior to stimulate apoptosis. We designed small interfering RNAs (siRNAs) for the knock down of c-Abl and tested whether the transfection into HeLa cells leads to reduced mRNA and protein levels of c-Abl. Quantitative real-time RT-PCR of c-Abl

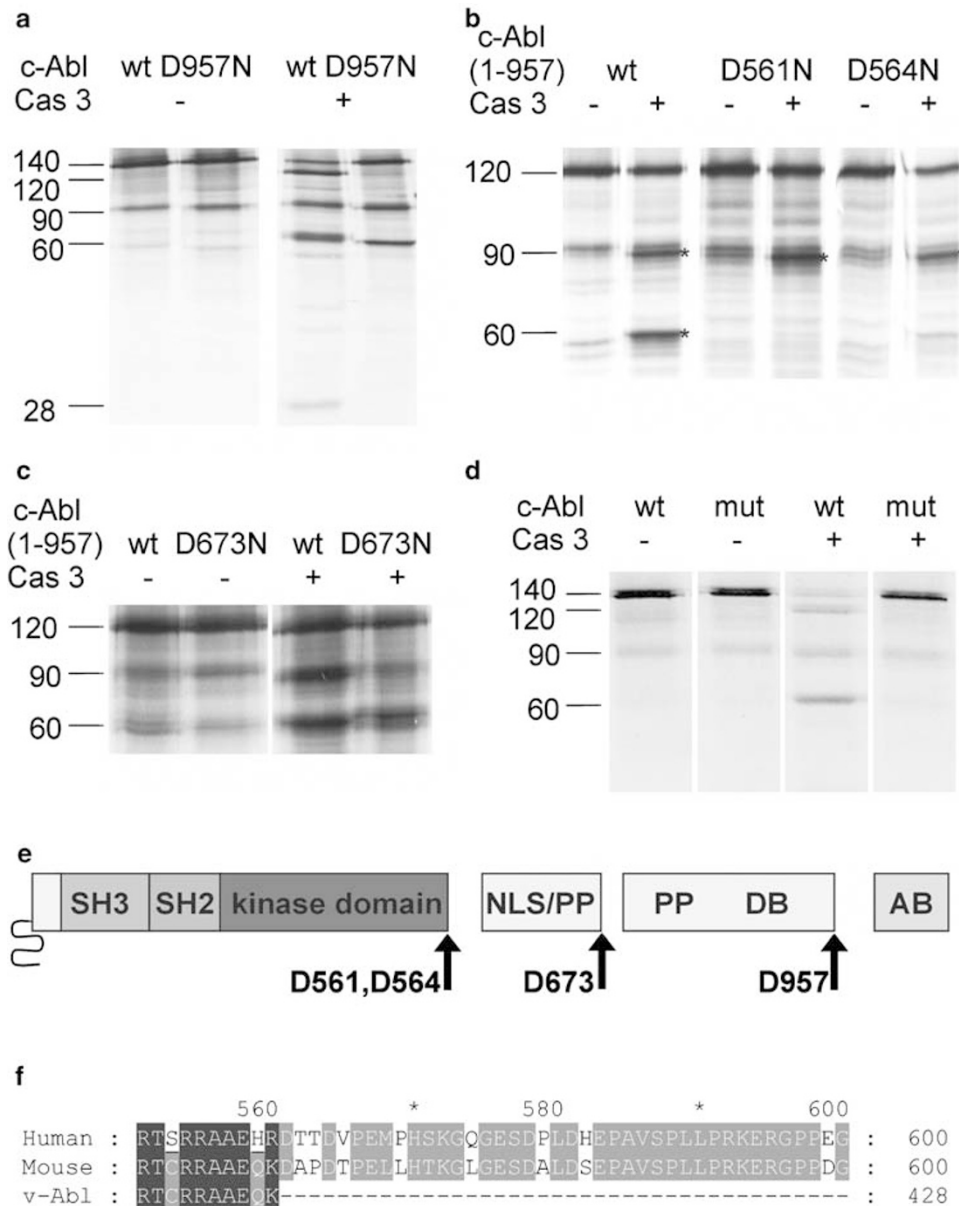


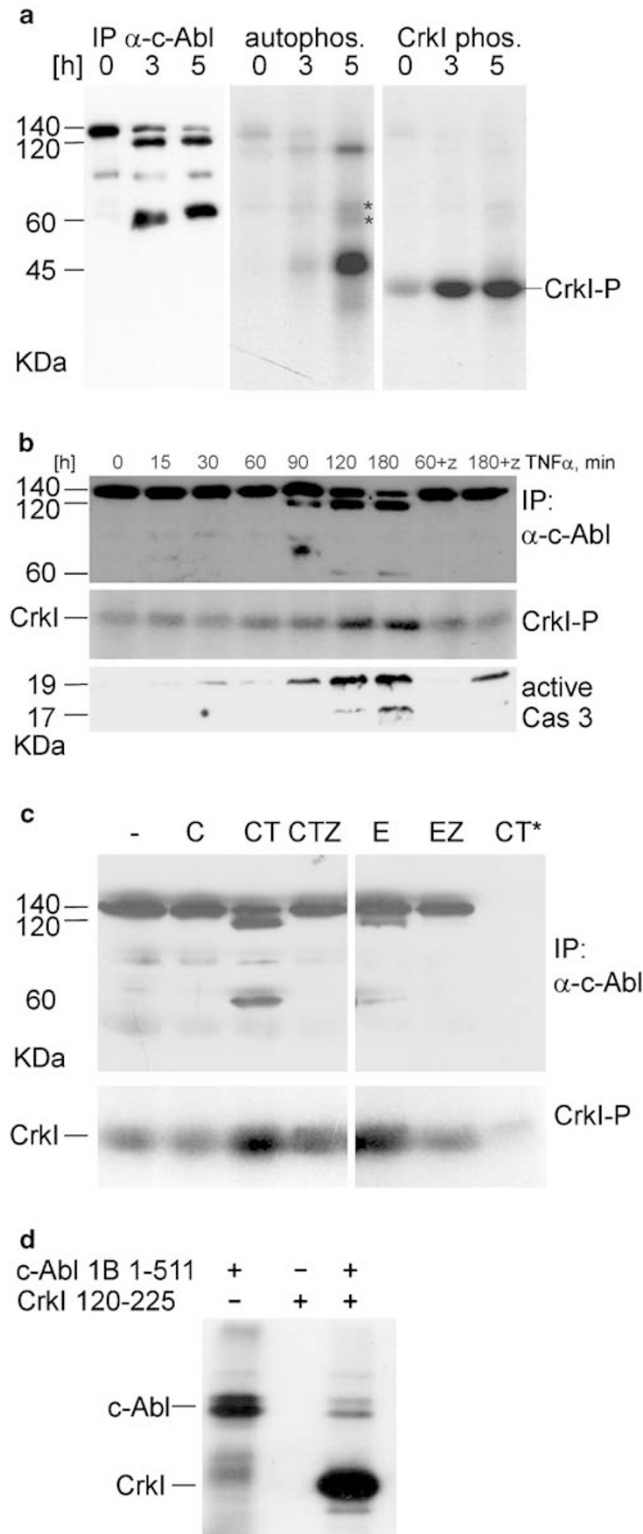
Figure 3 Identification of the caspase-cleavage sites in human c-Abl. (a)–(d) *In vitro* translated c-Abl 1B wild type (wt) or mutants thereof were either treated with recombinant caspase-3 (+) or buffer alone (–) for 1 h, separated by SDS-PAGE and analyzed by autoradiography. (a) wt, c-Abl 1B wt; D957N, c-Abl 1B D957N. (b) wt, c-Abl 1B aa 1–957; D561N, c-Abl 1B aa 1–957, D561N; D564N, c-Abl 1B aa 1–957, D564N. Cleavage products are indicated with an asterisk. (c) wt, c-Abl 1B aa 1–957; D673N, c-Abl 1B aa 1–957, D673N. (d) wt, c-Abl 1B; mut, c-Abl 1B D564N, D673N, D957N. (e) Schematic view of c-Abl caspase cleavage products. SH3, SH3-domain; SH2, SH2-domain; NLS, nuclear localization sequences; PP, PxxP motifs; DB, DNA-binding region; AB, actin-binding domains. (f) Sequence alignment of human c-Abl 1B and murine c-Abl IV compared to v-Abl p90 covering the region surrounding the first cleavage site in human c-Abl 1B (561DTTD564). The numbers above the sequence and on the right side indicate the amino-acid positions within the proteins

mRNA extracted from cells transfected with c-Abl siRNA consistently revealed more than 80% reduction in c-Abl mRNA levels when compared to control cells (Figure 5a). Likewise, immunoblots showed that c-Abl was significantly reduced in siRNA treated but not in control cells (Figure 5b). Knock down of c-Abl did not influence the onset of spontaneous apoptosis (Figure 5e), nor were these cells impaired in proliferation (not shown). However, the siRNA-treated cells turned out to be resistant to cisplatin-

induced apoptosis when compared to control cells (Figure 5c, e). In all, 30.6% of the cells transfected with the control siRNA and 9.4% of the cells transfected with siRNA directed against c-Abl displayed fragmented nuclei. However, no difference was observed when cells were treated with TNF α and cycloheximide to induce apoptosis (Figure 5c). Thus, c-Abl is required for only a specific pathway of apoptotic signaling, induced for example, by DNA damage but not TNF α -receptor ligation.

To investigate whether apoptosis induction is dependent on c-Abl cleavage, the wild type as well as a c-Abl mutant lacking all caspase cleavage sites were transfected in HeLa cells and analyzed for sensitivity to cisplatin or TNF α . The expression of

the constructs was equally efficient as tested by immunoblot (Figure 6a) and immunofluorescence assays (Figure 6b, c). Overexpression of the wild type, the triple mutant (Figure 6d) or the Src-homologue fragment (not shown), resulted in no significant increase in apoptosis. However, as the c-Abl knock downs (Figure 5), HeLa cells transfected with the triple cleavage mutant (Figure 6b, d) were resistant to cisplatin-induced apoptosis (14.9%), an effect which was not observed in wild-type transfected cells (33.6%; $P=0.0027$). TNF α -induced apoptosis was not decreased in the c-Abl wild type (44.1%) nor in triple mutant transfected cells (37.2%; $P=0.1$) (Figure 6c, d). These results suggest that c-Abl cleavage is required for apoptotic signaling induced by cisplatin, but not by TNF α .



Discussion

Owing to its extraordinary medical importance, c-Abl and its oncogenic forms v-Abl and BCR/Abl are among the most studied kinases in man. Structural alterations, a N-terminal fusion with the BCR protein in the case of the BCR/Abl or the viral Gag protein in case of v-Abl convert the tightly regulated cellular form of c-Abl into a constitutively active kinase with transforming potential. Strikingly, kinase activity is required not only for the transforming activity but also for the promotion of apoptosis since kinase-defective forms of c-Abl inhibited apoptosis in γ -irradiated MCF-7 cells.¹³ One of the major goals therefore was to identify the activating signals for c-Abl in apoptotic cells. Here, we show that c-Abl is a substrate for caspases, which cleave and activate c-Abl during apoptosis.

Caspases cleave human c-Abl in all tested cell lines into at least three fragments. Remarkably, each of the generated fragments comprised functional domains and cleavage occurred sequentially *in vivo*. First, a 120 and a 28 kDa fragment are generated by cleavage at D957. As a result, the C-terminal region containing the actin-binding domain and nuclear export sequence of c-Abl⁴ was separated from the Src-kinase and nuclear localization signals of the protein (Figure 3e). Then, after a lag of 1–2 h, the 60 kDa Src-kinase fragment was produced by cleavage at D561 or D564

Figure 4 Caspase cleavage correlates with the activation of c-Abl. (a), (b) U937 cells were treated with 50 ng/ml TNF α , 1 μ g/ml cycloheximide and z-VAD-fmk (z). After the indicated times lysates were prepared and subjected to immunoprecipitation with anti c-Abl antibody (K-12) for 3 h. The immunoprecipitates were washed and incubated with (A, Crkl phos. and B) or without (A, autophos.) purified recombinant Crkl in the presence of [³²P] γ ATP. Samples were separated by SDS page and analyzed by autoradiography. Active caspase-3 indicated by the appearance of the p17 fragment was monitored using antiactive caspase-3 antiserum. Immunoprecipitated c-Abl shown in A, B and C was detected in immunoblots using antibody 8e9 directed against the kinase domain. (c) U937 cells were incubated with 1 μ g/ml cycloheximide (C), 50 ng/ml TNF α (T), 50 μ M z-VAD-fmk (Z), 10 μ M etoposide (E) as indicated for 5 h and processed like in A. As a control for the antibody specificity, immunoprecipitation was performed with an antibody directed against the Flag-tag (CT*). (d) His-tagged purified recombinant c-Abl 1B aa 1–511 was subjected to phosphorylation reactions with or without Crkl, separated by SDS-PAGE and analyzed by autoradiography

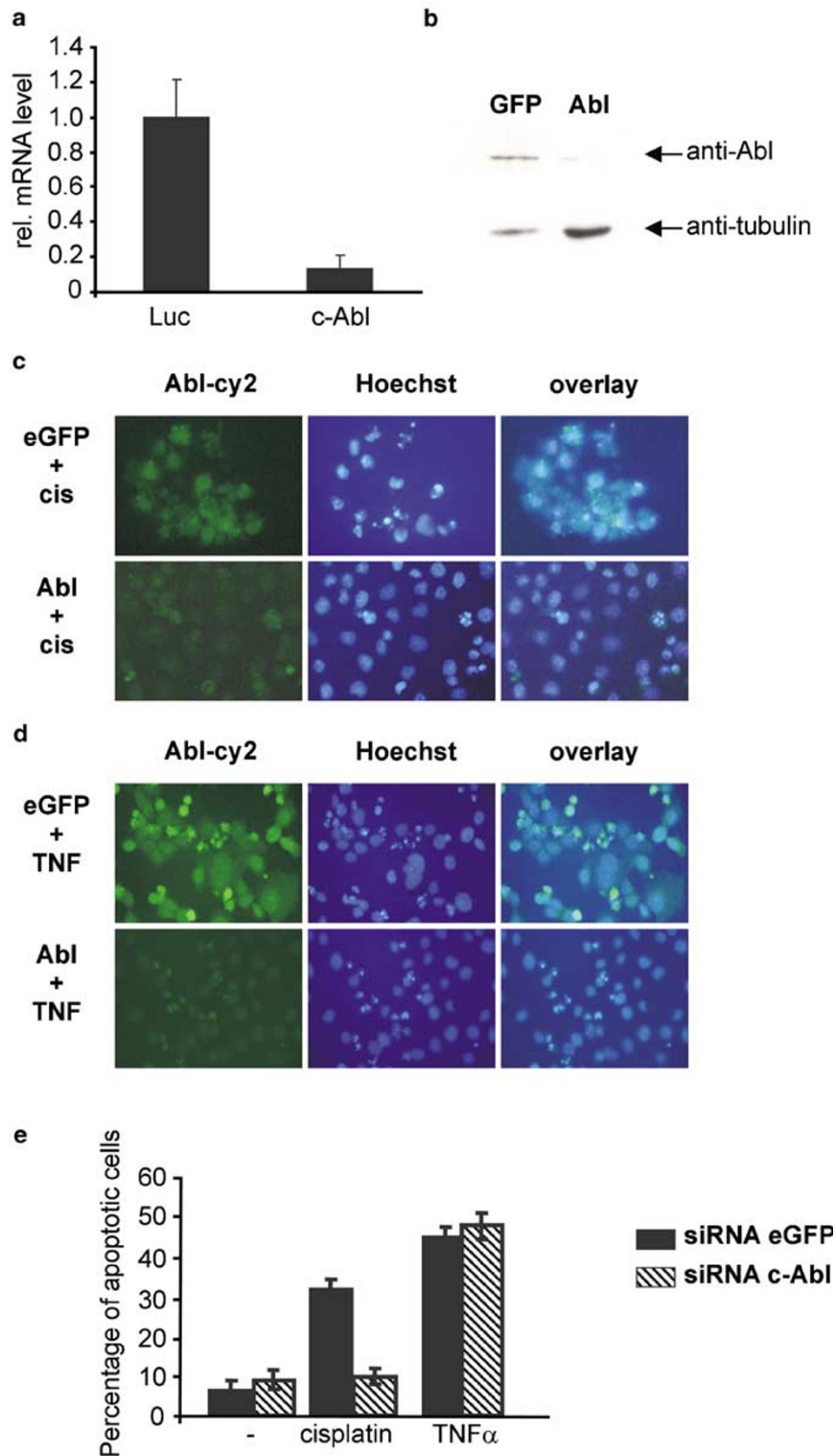


Figure 5 c-Abl is required for DNA damage-induced apoptosis. (a) HeLa cells were transfected with siRNAs against c-Abl and Luciferase as control. Knock down of c-Abl mRNA level was verified by real-time PCR. (b) Whole-cell lysates prepared from HeLa cells transfected with siRNA against c-Abl and eGFP were separated by SDS-PAGE and blotted onto PVDF membranes. The blots were probed with monoclonal antibodies against c-Abl and alpha-tubulin as described in Materials and Methods. (c,d) HeLa cells transfected with siRNAs against eGFP and c-Abl were induced to apoptosis with cisplatin (c) or TNF α (d). After induction the cells were fixed and costained for c-Abl and nuclei using a monoclonal antibody and Hoechst, respectively. Shown are the fluorescence images for c-Abl in the cy-2 channel (cy2) and Hoechst in the UV channel and the respective overlays from one representative field under a $\times 200$ magnification in the fluorescence microscope. (e) Quantitative evaluation of the experiments shown under C and D. Apoptotic and nonapoptotic cells from five random fields were counted under a fluorescence microscope ($\times 200$ magnification) and the percentage of apoptotic cells was calculated as mentioned in Materials and Methods. Shown are the means \pm S.D. of three independent experiments

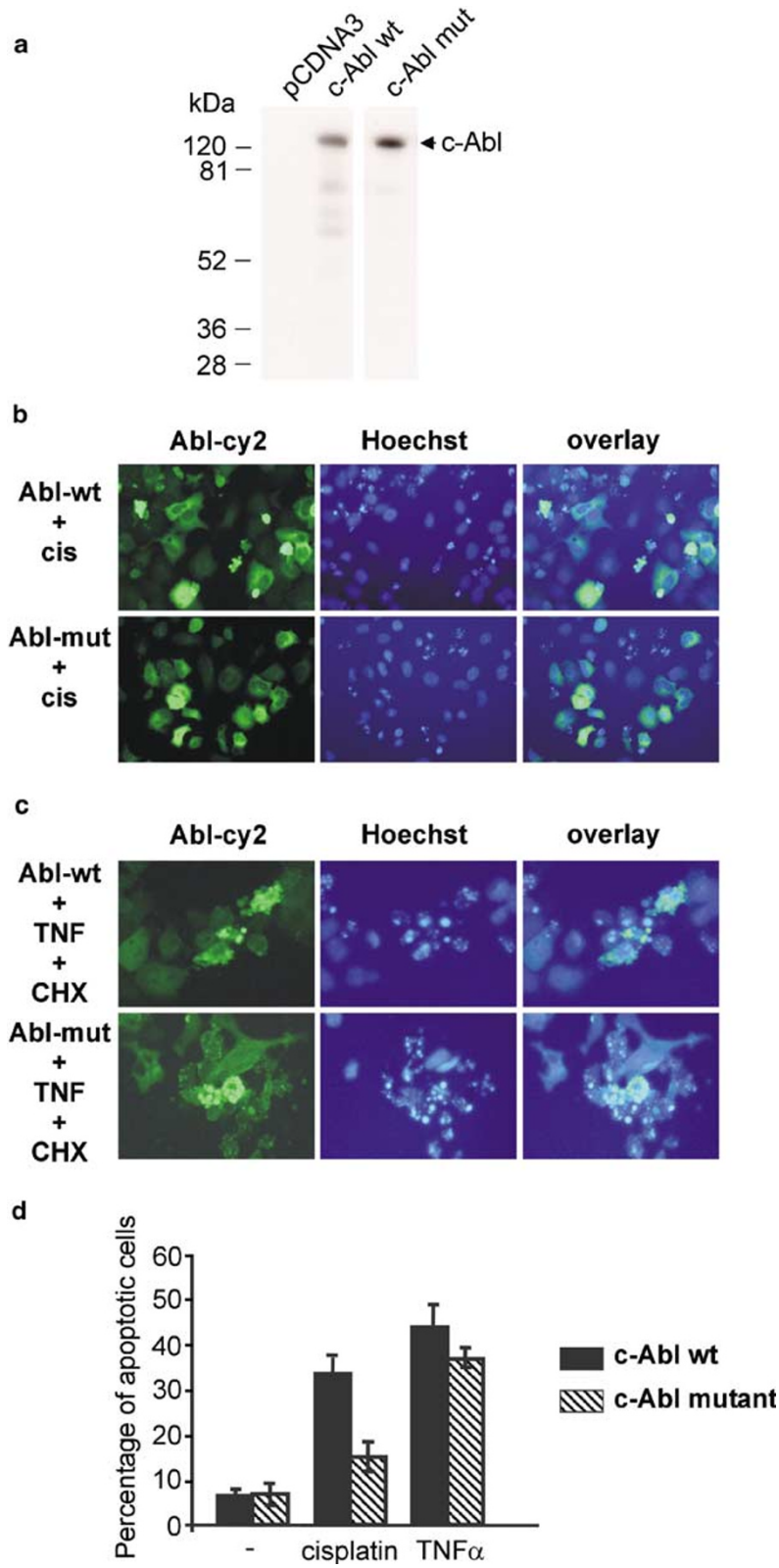


Figure 6 Caspase cleavage of c-Abl is required for DNA damage-induced apoptosis. **(a)** c-Abl wild type (c-Abl wt) and the triple mutant c-AblD564N, D673N, D957N (c-Abl mut) were transfected into HeLa cells and expression was monitored by immunoblot analysis. After transfection, the cells were challenged with either cisplatin **(b)** or TNF α **(c)** and apoptosis was monitored under the microscope. **(d)** Quantitative evaluation of the experiments shown under B and C. Only the transfected cells expressing various constructs of c-Abl were counted from five different fields and scored for apoptotic morphologies as described in Materials and Methods. The percentage of apoptotic cells from the transfected population was then calculated. Shown are the means \pm S.D. of three independent experiments

(Figure 1b). A 90 kDa fragment which could result from an initial cleavage after D561/D564 or D673 and cover the N- or C-terminal fragment of c-Abl respectively, was detected in *in vitro* cleavage assays but not in immunoblots of apoptotic cells. Therefore, caspases cleave c-Abl *in vivo* first after D957, then at D561/564 or D673. Since the initiator caspase-8 and -10 and the effector caspase-3 cleave c-Abl, sequential cleavage could depend on the activity of different caspases. Another possibility includes the re-localization of the partially cleaved kinase to a compartment of temporally lower caspase activity. It is also possible that interacting proteins transiently block cleavage of c-Abl.

Presently, we can only speculate on the functional consequences of the separation of the actin-binding domain, nuclear localization domains and the Src-homologue kinase. One possible function is the activation of the kinase activity. Since actin and nuclear localization domains are separated, it is to be expected that fragments relocalize during apoptosis to different cellular compartments. Moreover, many proteins have been shown to interact with different domains of the intact c-Abl. Whether these factors still interact with the domains contained in the cleaved fragments and the functional consequences must be reinvestigated.

c-Abl kinase precipitated from apoptotic lysates, but not from the nonapoptotic lysates was active in phosphorylating the c-Abl substrate Crkl. Phosphorylation of Crkl always correlated with the presence of the 60 kDa kinase fragment of c-Abl (Figure 4b). Furthermore, a recombinant fragment of c-Abl comprising the Src-homologue caspase cleavage product was fully active in phosphorylating Crkl *in vitro*, suggesting that the 60 kDa fragment contains the kinase activity. However, since we also observed phosphorylation of the 120 kDa fragment it is not clear whether the 120 kDa fragment is active and becomes autophosphorylated, or if it has to be processed to the 60 kDa fragment to be fully active. In conclusion, these data, and the inhibition of the TNF α - and etoposide-dependent activation of c-Abl by z-VAD-fmk, support our hypothesis of the direct activation of c-Abl by caspase cleavage.

Activation of kinases by caspase cleavage has been demonstrated before.²³ For example, caspases activate p21-activated kinase 2 by separating the autoinhibitory N-terminal fragment from the C-terminal kinase domain.¹⁹ The exact mechanism by which caspases activate c-Abl, however, remains to be shown. Activation of c-Abl depends on the conformation of a small inhibitory peptide contained in the N-terminus of c-Abl²⁴ which is not removed by caspase cleavage (N Machuy and T Rudel, unpublished). Caspase cleavage may therefore induce conformational changes of the N-terminus and thereby activate c-Abl. Another possibility is the release of c-Abl from cellular inhibitors which have been postulated by other groups to regulate c-Abl activity.^{25,26}

Knocking down c-Abl expression by RNA interference rendered the cells resistant to cisplatin- but not TNF α -induced apoptosis. However, overexpression of either the wild-type c-Abl or c-Abl fragments had no significant proapoptotic effect. Therefore, in the system under investigation, c-Abl is required but not sufficient for the initiation of apoptosis induced by

cisplatin. Moreover, overexpression of the caspase-resistant mutant of c-Abl, c-Abl (D564N, D673N, D957N) in HeLa cells efficiently blocked cisplatin-, but not TNF α -induced apoptosis. Recently, Barila and co-workers.³⁵ showed that a caspase-cleavage-resistant mutant of c-Abl inhibits Fas-induced apoptosis in the lymphoid cell line HuT78 suggesting a role of c-Abl also in receptor-induced apoptosis. Although we could not detect inhibition of receptor-initiated apoptosis by either c-Abl knock down or overexpression of cleavage-resistant mutant, the difference in our observation might depend on the specific death receptor pathway or the cell lines used.

One possibility of how the c-Abl cleavage mutant might block cisplatin-induced apoptosis is by displacing endogenous c-Abl. This assumption is in agreement with the localization of the overexpressed cleavage mutant which showed a similar distribution as the endogenous kinase. Since neither the inactive full-size kinase nor the inactive Src-homologue fragment generated by caspase cleavage were able to block cisplatin-induced apoptosis, we assume that cleavage of c-Abl has, in addition to activation, unknown consequences on c-Abl function.

At present, different possibilities could be considered of how cleavage of c-Abl transduces apoptotic signals. It might be that the interaction of p73 with the separated Src-homologue kinase fragment²⁷ and of p53 with the C-terminal fragments²⁸ might be relevant and we are currently investigating this possibility. There are several substrates of c-Abl implicated in apoptotic signaling. For example, phosphorylation of I κ B- α by c-Abl stabilizes the protein and prevents activation of the apoptosis antagonist NF κ B.²⁹ Mdm2, a protein implicated in p53 degradation, has been identified as a substrate of c-Abl. Phosphorylation of Mdm2 by c-Abl stabilizes p53 and promotes DNA-damage-induced apoptosis.³⁰ Additionally, c-Abl has been shown to directly bind, phosphorylate and activate MEKK-1 in response to DNA damage.³¹ Further, binding of c-Abl to the p85 subunit of PI3-kinase results in initiation of proapoptotic signals.³² Recently, caspase-2 has been identified as an initiator caspase for apoptotic signaling induced by DNA damage in some cell lines.³³ Whether c-Abl functions up- or downstream of caspase-2 is presently the subject of intensive research in our laboratory. Yet another possibility includes unknown factors that interact with fragments of the cleaved kinase. Whether the unknown c-Abl substrate found in c-Abl immunoprecipitates of apoptotic cells (Figure 4a) is such a factor remains to be shown.

In summary, we show that c-Abl is a substrate of caspases and that caspase cleavage of c-Abl is required for the transduction of apoptotic signals initiated by DNA damage. Since the cleavage-resistant forms of c-Abl efficiently blocked apoptosis, it is tempting to speculate that modified susceptibility to caspase cleavage has an impact on the role of c-Abl in cancer development. The lack of the caspase cleavage site in the viral homologue is only a first indication that lack of caspase cleavage may be of relevance for malignant transformation. The identification of caspase cleavage sites in the medically important c-Abl kinase may offer a novel approach to therapeutically convert the oncogenic forms of this kinase into its apoptotic counterpart.

Materials and Methods

Cell culture

SKW 6.4 and NIH-3T3 cells were maintained in DMEM tissue culture medium (Gibco BRL) supplemented with 10% fetal calf serum (Gibco BRL), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco BRL), 10 mM HEPES pH 7.5, and 4 mM L-glutamine at 37°C in 10% CO₂. Jurkat and U937 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco BRL) at 37°C in 5.0% CO₂.

Transfection and apoptosis induction

HeLa cells were seeded onto glass coverslips in 12-well plates (75 000/well) 1 day prior to transfection. Transfections with 0.5 µg of the various c-Abl constructs were performed using Lipofectamine-2000 (Invitrogen), according to the manufacturer's instructions. At 36 h post-transfection, the cells were induced to apoptosis for 15 h with 60 µM cisplatin or with 40 ng/ml of TNF-α with 2 µg/ml of cycloheximide for 6 h.

For RNA interference experiments, 50 000 cells/well in a 12-well plate were seeded at least 20 h prior to transfection. siRNAs against c-Abl (5'-AAAGGUGAAAAGCUCCGGGUC-3') and eGFP as negative control (5'-AAGUUCAGCGUGUCCGGCGAG-3') were transfected using the Transmessenger transfection kit (Qiagen) as per the manufacturer's instructions at a final concentration of 160 nM. At 2 days post-transfection, the nearly confluent cells were trypsinized and one-half of the cells were seeded onto coverslips in a 12-well plate for testing the response to apoptosis induction while the other half was used for Western blot analysis. Apoptosis was induced about 60 h after transfection under the same conditions as in the overexpression experiments.

Plasmid construction

The coding sequence of human c-Abl 1B (accession number X16416) was amplified from Jurkat RNA by RT-PCR and cloned into the mammalian expression vectors pCMV-Tag1, pCDNA3 and the bacterial expression vector pET28a. The caspase cleavage site-deficient mutants of c-Abl (D564N and/or D673N and/or D957N) were constructed by PCR. The c-Abl 1B coding region for the amino acids 1–957 (wt, D564N or D673N) and 1–511 were cloned into the *EcoRI*, *XhoI* sites of pET28a. The cDNA coding for amino acids 120–225 of Crkl was isolated by RT-PCR and cloned into the *EcoRI*, *XhoI* cleavage sites of the bacterial expression vector pGEX-4T3. The identity of all constructs was confirmed by sequence analysis.

Validation of RNAi by real-time PCR

In all, 20 000 cells/well were seeded in a 96 well plate one day prior to transfection. Transfection was performed with 0.25 µg siRNA directed against c-Abl or Luciferase (AACUUACGCUGAGUACUUCGA), GFP (AAGCUGACCCUGAAGUUCAUC) as controls, and 2 µl Transmessenger per well according to the manufacturer's instructions. After 48 h, RNA was isolated using the RNeasy[®] 96 BioRobot[®] 8000 system (Qiagen). The relative amount of c-Abl mRNA was determined by real-time PCR using Quantitect[™] SYBR[®] Green RT-PCR Kit (Qiagen) following the manufacturer's instructions. The expression level of c-Abl mRNA was normalized against the internal standard GAPDH. The following primers were used for RT-PCR: c-Abl 5': ATCACCATGAAGCACAAGCTG, c-Abl

3': GTAGGTCATGAACTCAGTGATG, GAPDH 5': GGTATCGTGGAAG-GACTCATGAC, GAPDH3': ATGCCAGTGAGCTTCCCGTTTCAG.

Immunoprecipitation

To prepare total cell lysates, cells were washed in ice-cold PBS, lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 1 mM NaVO₃, 10 mM Na-pyrophosphate, 1 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 mM Pefabloc, 10 µM pepstatin) for 30 min on ice and sonicated twice for 10 s. Lysates were cleared by centrifugation for 15 min at 13 000 rpm. Supernatants were incubated with agarose-coupled anti-Abl (K12, Santa Cruz Biotechnology) or OctaProbe (Santa Cruz Biotechnology) for 3 h.

SDS-PAGE and Western blot

For SDS-PAGE, cells were lysed in single detergent buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 mM Pefabloc, 10 µM pepstatin) for 30 min on ice and sonicated twice for 10 s. Lysates were cleared by centrifugation for 15 min at 13 000 rpm, subjected to SDS-PAGE and transferred to PVDF-membranes. For immunoblot analysis, membranes were blocked with 3% BSA in TBS with 0.5% Tween-20 for 90 min and incubated with anti-Abl (8e9, Pharmingen; C-19, Santa Cruz; Abi3, Oncogene science), anti-α Tubulin antibody (Sigma), or anticaspase-3 (D175, New England Biolabs). Antigen-antibody complexes were detected by horseradish peroxidase-coupled antibodies (Pharmingen) followed by enhanced chemiluminescence (NEN).

In vitro kinase assay

For *in vitro* kinase assays, immunoprecipitations were washed twice with RIPA buffer and kinase buffer w/o ATP (25 mM HEPES-NaOH pH 7.5, 5 mM MgCl₂, 4 mM MnCl₂). The beads were incubated at 30°C for 30 min in the presence of [³²P]ATP in kinase buffer supplemented with 30 µM ATP, with or without 5 µg of Crkl (AA 120–225), in a total volume of 40 µl. Kinase reactions were terminated by addition of SDS sample buffer and boiling. The proteins were separated by SDS-PAGE and analyzed by autoradiography.

Protein expression and purification

His-tagged c-Abl 1B (aa 1–511) and Gst-tagged Crkl (aa 120–225) were expressed in *E. coli* strain BL21 codon⁺ (Novagen). For cell lysis, bacteria were resuspended in ice-cold PBS with protease inhibitors and sonicated four times for 30 s. Bacterial debris was pelleted for 30 min at 13 000 rpm and Crkl or c-Abl fusion proteins were purified using glutathione-sepharose 4B (Pharmacia) or Ni-NTA (Qiagen), respectively, according to the suppliers user manual.

In vitro cleavage assays

cDNAs were translated *in vitro* using ³⁵S-labeled methionine with the TNT[®] system according to the manufacturer's instructions (Promega). 1 µl of the translation product was cleaved with 3 µl of active lysate from apoptotic Jurkat cells, ¹⁹ purified caspase-3 (100 ng), caspase-8 (500 ng), or caspase-10 (500 ng) in 20 µl of cleavage buffer (25 mM HEPES, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 mM Pefabloc, 10 µM pepstatin). Digestions were performed in the

absence or presence of 200 μ M z-Val-Ala-D-Asp-fluoromethylketone (z-VAD-fmk) for 1 h at 37°C. The cleavage mixture was separated by SDS-PAGE, and the gels were dried and exposed overnight to a BioMac™ MR film (Eastman Kodak Co.)

Immunofluorescence microscopy

Cells were fixed with 3% PFA for 30 min at room temperature, washed with PBS and permeabilized with 0.05% Triton-X100 in PBS. After blocking with 0.5% BSA, 1% goat serum and 0.05% Triton-X100 in PBS, cells were incubated with a rabbit polyclonal antibody (K12, Santa Cruz Biotechnology) diluted to 1 : 50 for overexpressed c-Abl or a monoclonal antibody (8e9, Pharmingen) diluted to 1 : 100 for endogenous c-Abl in 0.5% BSA and 1% goat serum in PBS for 60 min. Then, cells were washed and reprobed with Cy-2 labeled anti-rabbit or anti-mouse antibody diluted to 1 : 100 in the same buffer for 60 min. Finally, chromatin was stained using Hoechst dye (33258) and mounted on to glass slides with Moviole.

For overexpression experiments, only cells expressing various c-Abl constructs were counted from five different fields using fluorescence microscopy and checked for apoptotic phenotypes like chromatin fragmentation and plasma membrane blebbing. The percentage of apoptotic cells among the transfected population was then calculated. For siRNA experiments, cells from five random fields were counted under \times 200 magnification for each sample. The percentage of apoptotic cells was then calculated. On average, 100–120 cells were counted per sample in each experiment. The experiments were repeated three times. *t*-Test analysis was performed with Graph Pad Prism software.

2D-gel electrophoresis

Total lysates were separated by two-dimensional gel electrophoresis³⁴ and blotted on PVDF membranes followed by Western blot analysis with antibodies directed against the C-terminus (C-19, Santa Cruz Biotechnology, Santa Cruz, USA) of c-Abl. Isoelectric focusing rod gels with a diameter of 0.9 mm were used for the first dimension. SDS-polyacrylamide gels with 10% (w/v) acrylamide and 0.2% bisacrylamide were used for the second dimension.

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