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Phosphorylation of IκB-α Inhibits Its Cleavage by Caspase CPP32 *in Vitro**

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IκB proteins function as direct regulators of Rel/NF-κB transcription complexes. We show that the cell-death protease CPP32 (caspase-3) *in vitro* specifically cleaved chicken and human IκB-α at a conserved Asp-Ser sequence. This cleavage site appears to be identical to the site at which chicken IκB-α is cleaved *in vivo* in temperature-sensitive v-Rel-transformed chicken spleen cells undergoing apoptosis. Other caspases, namely interleukin-1β-converting enzyme (caspase-1) and Ich-1 (caspase-2), did not cleave IκB-α. CPP32 also cleaved mammalian IκB-β *in vitro* at the analogous Asp-Ser sequence. Cleavage of IκB-α by CPP32 was blocked by serine phosphorylation of IκB-α. Cleavage of IκB-α by a CPP32- like protease could generate a constitutive inhibitor of Rel transcription complexes. This report provides evidence for a direct biochemical interaction between the NF-κB signaling pathway and a cell-death protease signaling pathway.

Apoptosis is a form of regulated programmed cell death that is involved in normal development and organ homeostasis (reviewed in Ref. 1). One cellular pathway leading to apoptosis involves a family of related cysteine proteases (caspases) first defined by the *Caenorhabditis elegans* cell-death protease CED-3 and the interleukin-1β-converting enzyme (ICE)[1] (reviewed in Refs. 2 and 3). Although all caspases cleave C-terminal to Asp residues, individual proteases show distinct substrate specificities *in vitro* and can be divided into subfamilies based on substrate preference (3, 4). The caspase CPP32 shows a substrate specificity similar to that of CED-3, and CPP32 appears to be an important cell-death protease in vertebrates. For example, mice with a disruption of the gene encoding CPP32 have reduced neural cell death (5). It is likely that in many cell types specific cell-death proteases are activated in a sequential manner to lead to cell death (6). Thus, one class of *in vivo* substrates for the cell-death proteases includes the caspases themselves. In addition, several other proteins have been identified as substrates for caspases (reviewed in Ref. 3).

The IκB proteins comprise a conserved family of proteins that act as regulators of the Rel/NF-κB family of transcription factors (reviewed in Ref. 7). IκB proteins are structurally related in that they all have a central core of ankyrin repeats that are essential for interaction with Rel complexes. Interaction of an IκB protein with a Rel complex usually results in retention of the Rel complex in the cytoplasm and inhibition of the DNA binding activity of the Rel complex.

In the best characterized case, IκB-α interacts with and inhibits the activity of NF-κB. In response to a variety of signals, IκB-α becomes phosphorylated on two Ser residues in its N-terminal regulatory domain (8–10). This N-terminal phosphorylation leads to ubiquitination of IκB-α at nearby Lys residues, thereby targeting IκB-α for cleavage by the proteasome (11–13). The free NF-κB complex can then enter the nucleus and affect gene transcription.

We have previously shown that IκB-α undergoes a specific N-terminal cleavage in chicken spleen cells transformed by a temperature-sensitive mutant of the retroviral oncoprotein v-Rel when these cells are induced to undergo apoptosis by a shift to the nonpermissive temperature (14). This observation led us to suggest that IκB-α might be a direct substrate for a cell-death protease, which could cleave IκB-α at a conserved Asp near the N terminus (Fig. 1*A*; Ref. 15).

In this report, we show that IκB-α is a substrate for CPP32 *in vitro*. Cleavage of IκB-α by CPP32 could create a dominant inhibitor of Rel transcription complexes.

EXPERIMENTAL PROCEDURES

Cells—Chicken spleen cell lines transformed by ts mutant v-G37E were cultured in Temin's modified Eagle's medium containing 20% fetal bovine serum as described previously (14).

Plasmids and in Vitro Mutagenesis—Site-directed mutagenesis of p40 was performed using the method of Kunkel (16), as described previously (17). An *Eco*RI to *Hin*cII fragment from an IκB-α/p40 cDNA was first subcloned into M13mp19. The following oligonucleotides were used on single-stranded DNA templates to create the indicated IκB-α/p40 mutants: D35A, 5′-GACGACCGCCACGCCAGCGGGCTGGAC-3′; D39A, 5′-GACCGCCACGACAGCGGGCTGGCCTCCATG-3′; S36A-/S40A, 5′-CGCCACGACGCCGGGCTGGACGCCATGAAG-3′; S36-E/S40E, 5′-GACCGCCACGACGAAGGGCTGGACGAACTGAAG-3′. The D35A mutation introduced a *Bst*XI site that was used in screening for other mutations. All mutations were confirmed by DNA sequencing.

To create *in vitro* expression vectors for IκB-α/p40, human IκB-α, and mouse IκB-β, cDNAs were subcloned into pGEM4. An *Eco*RI to *Hin*cII fragment containing wild-type p40 sequences was subcloned into pGEM4 digested with *Eco*RI and *Hin*cII; expression vectors for mutant IκB-α/p40 proteins were made by replacing wild-type sequences with appropriate mutant fragments. Wild-type and D31A human IκB-α inserts were subcloned as *Kpn*I to *Not*I/Klenow-treated fragments into pGEM4 digested with *Kpn*I and *Hin*cII. An *Eco*RI to *Xho*I fragment from a mouse IκB-β cDNA was subcloned into pGEM4 digested with *Eco*RI and *Sal*I.

In Vitro Cleavage by Caspases—All *in vitro* translations were performed in the TNT-coupled wheat germ extract (Promega) using SP6 polymerase in the presence of Tran^{35}S-label (Amersham Corp.). Cleav-

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[1] The abbreviations used are: ICE, interleukin-1β-converting enzyme; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis.

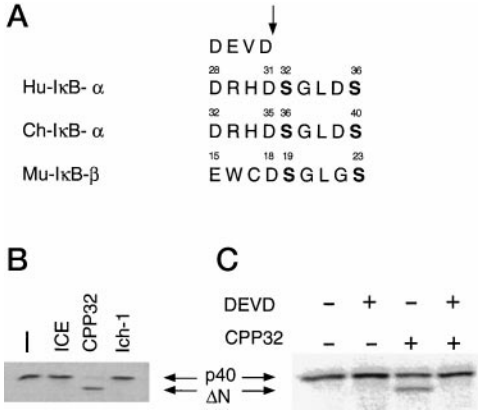

FIG. 1. **Cleavage of chicken IκB-α (p40) by CPP32.** *A*, shown is the conserved region of signal-induced serine (*bold S*) phosphorylation in the indicated IκB proteins (*Hu*, human; *Ch*, chicken; *Mu*, murine); relevant amino acid residues are indicated above each sequence. DEVD is the sequence of a potent inhibitor of CPP32, and the predicted cleavage site C-terminal to the aspartate residue is indicated by the *arrow*. *B*, specific cleavage of p40 by CPP32. *In vitro* translated, radiolabeled p40 was incubated without (−) or with (+) the indicated proteases. *C*, p40 was incubated without (−) or with (+) CPP32 and in the absence (−) or presence (+) of the tetrapeptide inhibitor Ac-Asp-Glu-Val-Asp-aldehyde (*DEVD*). In *B* and *C*, samples were analyzed by SDS-PAGE followed by autoradiography and phosphorimaging, respectively. The positions of full-length p40 and cleaved p40 (Δ*N*) are indicated by *arrows*.

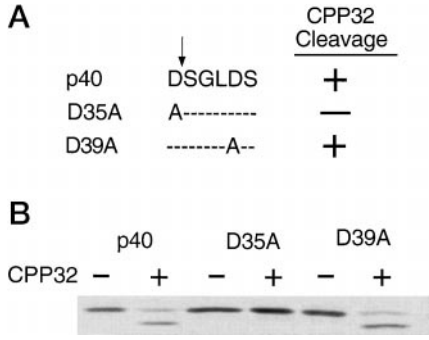

FIG. 2. **Identification of the site of CPP32 cleavage in p40.** *A*, sequences of site-directed mutants in p40. *B*, the indicated p40 proteins were translated *in vitro* and incubated without (−) and with (+) CPP32. Samples were separated by SDS-PAGE, and proteins were detected by autoradiography.

age of *in vitro* translated proteins by individual caspases was performed as described previously (4). Briefly, *in vitro* translated substrate proteins were incubated in CED-3 buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, 5% glycerol) with approximately 40 ng of a given bacterially produced and purified enzyme for 2–6 h at 30 or 37°C. Where indicated, the CPP32 inhibitor Ac-Asp-Glu-Val-Asp-aldehyde (DEVD; Bachem) was included at a concentration of 50 μM. Phosphorylation of *in vitro* translated p40 was carried out with purified IκB kinase as described previously (18). Phosphorylated p40 was dephosphorylated by treatment with calf intestinal phosphatase (Boehringer Mannheim) for 45 min at 30°C. Samples were separated on SDS-PAGE, and ^{35}S-labeled proteins were detected by autoradiography or using a phosphorimager (Bio-Rad).

Western Blotting—Western blotting was performed as described previously (14) using anti-Rel primary antiserum (1:500) (19) or an anti-p40 monoclonal antibody (anti-ANK; HY95) (1:2500) (14). The appropriate secondary antiserum was added, and complexes were detected by enhanced chemiluminescence (Amersham) and autoradiography.

RESULTS

Chicken IκB-α (p40) Is a Substrate for CPP32 in Vitro—To determine whether chicken IκB-α (called p40 hereafter) could serve as a direct substrate of a caspase, *in vitro* translated p40 was incubated with bacterially expressed and purified ICE, CPP32, and Ich-1, which represent apparently distinct classes of enzymes within the caspase family (2, 4). p40 was specifically cleaved by CPP32, but not by ICE or Ich-1 (Fig. 1*B*). Cleavage of p40 *in vitro* by CPP32 was inhibited by Ac-Asp-Glu-Val-Asp-aldehyde (DEVD), a specific peptide inhibitor of CPP32-like proteases (Fig. 1, *A* and *C*). Thus, p40 is a substrate of CPP32 *in vitro*.

CPP32 Cleaves p40 between Asp-35 and Ser-36—CPP32-like proteases cleave C-terminal to Asp residues that are frequently followed by Gly, Ser, or Ala and that are often in the consensus sequence Asp-*X*-*X*-**Asp**-Gly/Ser/Ala (3). There is a potential CPP32 cleavage site (Asp-Arg-His-**Asp**-Ser-Gly-Leu-Asp-Ser, aa 32–40; Fig. 1*A*) between aa 35 and 36 of p40. To determine if this was the site of CPP32 cleavage in p40, mutants with site-directed changes in p40 were incubated with CPP32 (Fig. 2, *A* and *B*). Mutant D35A, in which the predicted Asp cleavage site was changed to an Ala, was not detectably cleaved by CPP32. In contrast p40 mutant D39A was cleaved by CPP32 to a similar extent as wild-type p40. The cleaved form of p40 is not recognized by a monoclonal antibody directed against the N terminus of p40, indicating that CPP32 cleaved near the N terminus of p40 *in vitro* (data not shown; Ref. 14). These results indicate that CPP32 cleaved p40 between Asp-35 and Ser-36.

In Vitro Cleaved p40 Co-migrates on SDS-Polyacrylamide Gels with in Vivo Cleaved p40—We previously showed that p40 is cleaved near its N terminus in chicken spleen cells transformed by ts v-Rel mutant v-G37E when these cells are induced to undergo apoptosis by a shift to the nonpermissive temperature (14, 15). As shown in Fig. 3, *A* and *B*, the proteolyzed form of p40 generated by *in vitro* cleavage with CPP32 co-migrates on SDS-polyacrylamide gels with the major N-terminally truncated form of p40 seen in ts v-Rel-transformed cells undergoing apoptosis. This result suggests that the N-terminal cleavage of p40 *in vitro* by CPP32 is identical to the cleavage of p40 that occurs in ts v-G37E-transformed chicken spleen cells undergoing apoptosis.

Phosphorylation at Sites of Signal-induced Phosphorylation Blocks the Ability of Chicken p40 to Serve as a Substrate for CPP32—Ser-36 and Ser-40, which are located just beyond Asp-35 (the site of CPP32 cleavage) in p40, are sites of signal-induced phosphorylation (8–10). Phosphorylation at these Ser residues can be mimicked by Glu substitutions at these sites (8), and IκB-α can be phosphorylated *in vitro* at these Ser residues by a purified MEKK1-activated kinase from HeLa cells (18). To determine whether phosphorylation at these Ser residues affects the ability of p40 to serve as a substrate for CPP32, we tested whether p40 double mutant S36E/S40E as well as *in vitro* phosphorylated p40 could be cleaved by CPP32 *in vitro* (Fig. 4). Neither the S36E/S40E mutant nor wild-type p40 phosphorylated at Ser-36 and Ser-40 *in vitro* was cleaved by CPP32. In contrast, p40 mutant S36A/S40A (Ser → Ala) was cleaved by CPP32 to the same extent as wild-type nonphosphorylated p40. Treatment of phosphorylated p40 with calf intestinal phosphatase rendered p40 susceptible to cleavage by CPP32. Taken together, these results suggest that signal-induced phosphorylation of IκB-α *in vivo* would block its ability to be cleaved by a CPP32-like protease(s).

Other Vertebrate IκB Proteins Can Also Be Cleaved by CPP32—The Asp-Ser sequence at aa 35–36 of chicken IκB-α/p40 is conserved in mammalian IκB-α and IκB-β (aa 31–32 in human IκB-α and aa 19–20 in mouse IκB-β; Fig. 1*A*). To determine whether these mammalian IκB proteins could also serve as substrates for CPP32, *in vitro* translated human IκB-α and mouse IκB-β were incubated with CPP32 (Fig. 5). Each mammalian IκB protein was cleaved by CPP32 to a size consistent with cleavage at this Asp-Ser sequence. Furthermore, human IκB-α mutant D31A, containing a mutation at the pre-

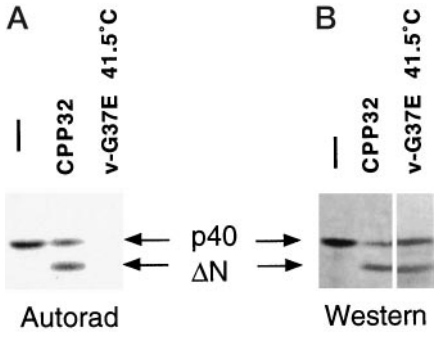

FIG. 3. **p40 cleaved with CPP32 *in vitro* co-migrates with p40 cleaved in ts v-Rel-transformed cells undergoing apoptosis.** *In vitro* translated, radiolabeled p40 (*first two lanes*) was incubated without (−) or with (+) CPP32. The *third lane* of this gel contains an unlabeled lysate from ts v-G37E-transformed spleen cells that had been shifted to the nonpermissive temperature for 48 h. Samples were transferred to a filter that was exposed to film directly (*A*) or probed by Western blotting with an anti-p40 monoclonal antibody (*B*). To equalize the images, the *first two lanes* in *B* are from a longer exposure than in the *third lane*. p40 indicates full-length p40; Δ*N* indicates the N-terminally cleaved form of p40.

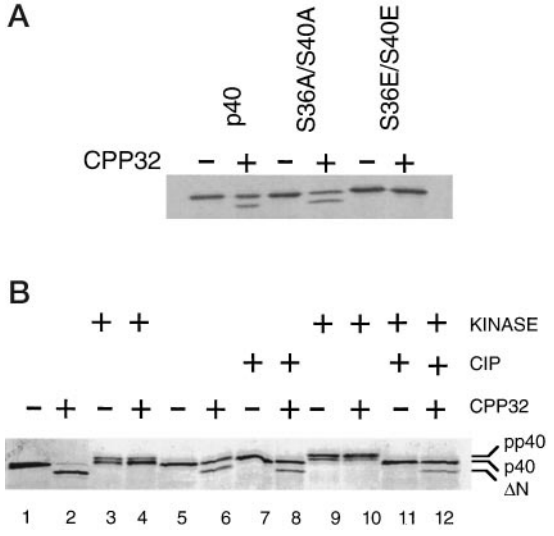

FIG. 4. **Phosphorylated p40 is not a substrate for CPP32.** *A*, wild-type p40 and the indicated p40 mutants were incubated without (−) or with (+) CPP32. *B*, p40 was translated *in vitro* and, where indicated, treated with MEKK1-activated IκB-α kinase (*lanes 3, 4*, and *9–12*) and/or calf intestinal phosphatase (*lanes 7, 8, 11*, and *12*). The phosphorylated form of p40 (*pp40*) migrates as a doublet with a slower mobility than nonphosphorylated p40. As indicated, samples were then incubated without (−) or with (+) CPP32. All samples were analyzed as described for Fig. 1.

dicted Asp cleavage site, was not cleaved by CPP32. Thus, mammalian IκB proteins can also be cleaved by CPP32 *in vitro*.

DISCUSSION

In this report, we describe biochemical evidence for a link between an apoptosis pathway and the Rel/NF-κB signal transduction pathway. Specifically, we have shown that IκB proteins can serve *in vitro* as direct substrates for the cell-death protease CPP32. In addition, the chicken p40 cleavage product generated by *in vitro* cleavage with CPP32 appears to be identical to that generated *in vivo* in temperature-sensitive v-Rel-transformed cells undergoing apoptosis (14, 15). CPP32 is expressed in these cells,[2] indicating that CPP32 may be the *in vivo* cleaving activity; however, we cannot exclude the possibility that a related protease cleaves p40 in these cells.

[2] M. Barkett and T. D. Gilmore, unpublished results.

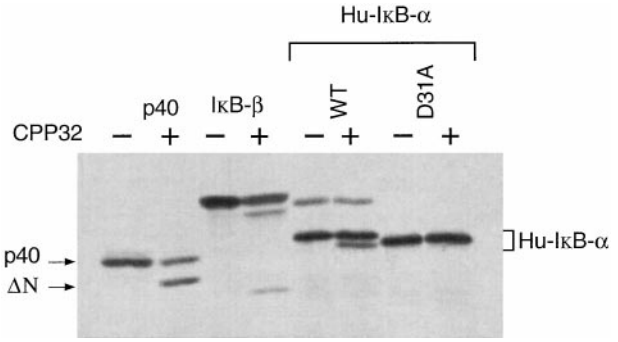

FIG. 5. **Cleavage of mammalian IκB proteins by CPP32.** The indicated radiolabeled proteins were incubated without (−) or with (+) CPP32, and samples were analyzed as described for Fig. 1.

Three observations indicate that cleavage of p40 is not essential for the apoptosis that occurs in ts v-Rel-transformed cells shifted to the nonpermissive temperature. First, cleavage of p40 is a late event in ts v-Rel-transformed spleen cells undergoing apoptosis (14). Second, Bcl-2 blocks apoptosis in these cells but does not block cleavage of p40 (15). Third, CrmA (a cowpox virus-encoded inhibitor of caspases) blocks N-terminal cleavage of p40 in these cells but does not block apoptosis (15).

Our results indicate that cleavage of IκB-α by a CPP32-like protease is distinct from signal-induced, proteasome-mediated cleavage of IκB-α. Namely, signal-induced cleavage of IκB-α requires phosphorylation at two N-terminal Ser residues, whereas phosphorylation or mutations that mimic phosphorylation at these Ser residues block the ability of CPP32 to cleave IκB-α. Therefore, we appear to have identified a novel pathway for proteolysis of IκB-α.

Overexpression of an N-terminally deleted form of IκB-α, which is resistant to ubiquitination-based degradation, prevents activation of NF-κB (20). Therefore, cleavage of IκB-α by a CPP32-like protease could create what is sometimes called a super-repressor form of IκB-α (20). That is, cleavage by CPP32 would block the ability of IκB-α to undergo signal-induced degradation by removing the sites of signal-induced ubiquitination and by likely disrupting the ability of IκB-α to become phosphorylated at critical Ser residues. Therefore, cleavage of IκB-α by CPP32 would block activation of Rel/NF-κB complexes and their responsive genes.

Activation of the tumor necrosis factor receptor has recently been shown to induce two conflicting pathways, one leading to CPP32-mediated apoptosis and one leading to activation of NF-κB, which is anti-apoptotic (20–23). Inhibition of NF-κB activation by a noninducible form of IκB-α renders cells more susceptible to tumor necrosis factor-induced apoptosis (20, 21, 23). Therefore, cleavage of IκB-α by a CPP32-like protease may act to facilitate, rather than effect, apoptosis.

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