Communication

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 vitro 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 proteosome (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. 1A; 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 EcoRI to HindIII 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'-GACGACCGCCACGCCAGCGGGCTGGACGCCATGAAG-3'; D39A, 5'-GACGACCGCCACGCCAGCGGGCTGGACGCCATGAAG-3'; S36-A/S40A, 5'-CGGACGACCGCCACGCCAGCGGGCTGGACGCCATGAAG-3'; S36-E/S40E, 5'-GACGACCGCCACGCCAGGCGGCTGGACGCCATGAAG-3'. The D35A mutation introduced a BstXI 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 EcoRI to HindIII fragment containing wild-type p40 sequences was subcloned into pGEM4 digested with EcoRI and HindIII; 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 KpnI to NotI/Klenow-treated fragments into pGEM4 digested with KpnI and HindIII. An EcoRI to XhoI fragment from a mouse IκB-β cDNA was subcloned into pGEM4 digested with EcoRI and SacI.

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 Tran32P-label (Amersham Corp.). Cleav-
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-α-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 by SDS-PAGE, and proteins were detected by autoradiography or phosphorimaging, respectively. The positions of full-length p40 and cleaved p40 (ΔN) are indicated by arrows.

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 IκB-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 IκB-1 (Fig. 1A). Cleavage of p40 in vitro by CPP32 was inhibited by Ac-Asp-Glu-Val-Asp-α-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-Val-Leu-Ser, aa 32–40; Fig. 1A) 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 terminal 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. 1A). 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-
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, indicating that CPP32 may be the in vitro cleaving activity; however, we cannot exclude the possibility that a related protease cleaves p40 in these cells.

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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IκB and CPP32 Cleavage

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