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

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 cytosine proteases (caspases) first defined by the Caenorhabditis elegans cell-death protease CED-3 and the interleukin-1β-converting enzyme (ICE) (I) (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 proteases 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 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 HincII 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'-GACCGCCACGACGAAGGGCTGGACGAACTGAAG-3'; D39A, 5'-GACGACCGCCACGCCAGCGGGCTGGACGC-3'; D39A, 5'-GACGACCGCCACGCCAGCGGGCTGGACGC-3'; S36A, 5'-GACGACCGCCACGCCAGCGGGCTGGACGC-3'; S36A, 5'-GACCGCCACGCCAGCGGGCTGGACGC-3'; S36A, 5'-GACCGCCACGCCAGCGGGCTGGACGC-3'; S36A, 5'-GACCGCCACGCCAGCGGGCTGGACGC-3'; S36A, 5'-GACGACCGCCACGCCAGCGGGCTGGACGC-3'; S36A, 5'-GACGACCGCCACGCCAGCGGGCTGGACGC-3'.

The D35A mutation introduced a KpnI and XhoI 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 HincII fragment containing wild-type p40 sequences was first subcloned into pGEM4 digested with EcoRI and HincII; 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 HincII. An EcoRI to XhoI fragment from a mouse IκB-β cDNA was subcloned into pGEM4 digested with EcoRI and Sall.

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 Trun-35S-label (Amersham Corp.). Cleav-
C. 

**Fig. 1. Cleavage of chicken I kB-α (p40) by CPP32.** A, shown is the conserved region of signal-induced serine (bold S) phosphorylation in the indicated I kB 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 (ΔN) are indicated by arrows.

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 I kB 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 32P-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 antiseraum (1:500) (19) or an antip40 monoclonal antibody (anti-AK, HY975) (1:2500) (14). The appropriate secondary antiseraum was added, and complexes were detected by enhanced chemiluminescence (Amersham) and autoradiography.

**RESULTS**

**Chicken I kB-α (p40) Is a Substrate for CPP32 in Vitro—** To determine whether chicken I kB-α (called p40 hereafter) could serve as a direct substrate of a caspase, in vitro translated p40 was incubated with bacterially expressed and purified I CE, CPP32, and I ch-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 I ch-1 (Fig. 1B). Cleavage of p40 in vitro by CPP32 was inhibited by Ac-Asp-Glu-Val-Asp-aldehyde (DEVD), a specific peptidase 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 conserved sequence Asp-X-X-Asp-Gly-Ser/Ala (3). There is a potential CPP32 cleavage site (Asp-Arg-His-Asp-Ser-Gly-Leu-Ser-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 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 I kB-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 I kB-α in vitro would block its ability to be cleaved by a CPP32-like protease(s).

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

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