The Large Subunit of the DNA Replication Complex C (DSEB/RF-C140) Cleaved and Inactivated by Caspase-3 (CPP32/YAMA) during Fas-induced Apoptosis*

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We report the identification of the large subunit of the DNA replication factor, DSEB/RF-C140, as a new substrate for caspase-3 (CPP32/YAMA), or a very closely related protease activated during Fas-induced apoptosis in Jurkat T cells. DSEB/RF-C140 is a multifunctional DNA-binding protein with sequence homology to poly-(ADP-ribose) polymerase (PARP). This similarity includes a consensus DEVD/G cleavage site for caspase-3. Cleavage of DSEB/RF-C140 is predicted to occur between Asp706 and Gly707, generating 87-kDa and 53-kDa fragments. An antiserum raised against the amino-terminal domain of DSEB/RF-C140 detects a new 87-kDa protein in Jurkat T cells in which apoptosis is activated by a monoclonal antibody to Fas. This cleavage occurs shortly after PARP cleavage. In vitro translated DSEB/RF-C140 is specifically cleaved into the predicted fragments when incubated with a cytoplasmic extract from Fas antibody-treated cells. Proteolytic cleavage was prevented by substituting Asp706 by an alanine in the DEVD/G caspase-3 cleavage site. The cleavage of DSEB/RF-C140 is prevented by iodoacetamide and the specific caspase-3 inhibitor, tetrapeptide aldehyde Ac-DEVD-CHO, but not by the specific ICE (interleukin-1-converting enzyme) inhibitors: CrmA and Ac-YVAD-CHO, indicating that the protease responsible for the cleavage of DSEB/RF-C140 during Fas-induced apoptosis in Jurkat cells is caspase-3, or a closely related protease. This conclusion is reinforced by the fact that recombinant caspase-3 but not caspase-1 reproduced the “in vivo” cleavage. Inasmuch as the cleavage of DSEB/RF-C140 separates its DNA binding from its association domain, required for replication complex formation, we propose that such a cleavage will impair DNA replication. Recent in vitro mutagenesis support this proposal (Uhlmann, F., Cai, J., Gibbs, E., O’Donnel, M., and Hurwitz, J. (1997) J. Biol. Chem. 272, 10058–10064).

Programmed cell death or apoptosis is well recognized as a physiological process essential for normal tissue homeostasis within multicellular organisms (1–4). Cells undergoing apoptosis are characterized by nuclear condensation, DNA fragmentation, contraction of the cytoplasm, and blebbing of the plasma membrane (5, 6). Some of the molecular processes responsible for the morphological changes observed during apoptosis in mammalian cells have been elucidated in the invertebrate Caenorhabditis elegans (7). Activation of a family of aspartate-specific cysteine proteases, initially defined by their homology to the C. elegans protein CED3, and homolog to the mammalian interleukin-1-converting enzyme (ICE),1 appear to be critical for apoptosis to occur (8–10). Due to a frenetic pace in the identification of new members of this protease family, a nomenclature committee has recently recommended the use of the term caspase to refer to those cysteine proteases that belong to the ICE/CED-3 family (49). Signals generated from inside the cells due to cellular damage or genetically determined space-time switches during development, and the presence or absence of cell-specific extracellular signals, can irreversibly trigger the biochemical cascade that results in programmed cell death. Extracellular signals such as the occupation of the Fas/APO-1 receptor by the Fas ligand or an antibody with agonist properties constitutes a well-characterized model in which to study the events responsible for the initiation and execution of the apoptotic program (11). Fas-dependent apoptosis in mammalian cells resembles programmed cell death in C. elegans, inasmuch as both require the activation of ICE/CED-3 proteases (12) (caspases). The sequential activation of ICE (caspase-1)-like and CPP32/YAMA (caspase-3)-like proteases during apoptosis has been suggested (13, 16). Although current studies have focused on the elucidation of the biochemical pathways by which the occupation of the Fas-receptor results in the activation of the caspase (ICE/CED-3) protease cascade (14, 15) and the identification of the sequential activation of specific members of this protease family (16), less attention has been directed toward the identification of the cellular substrates for such proteases. Although several proteins have been identified as specific substrates for caspase proteases during apoptosis (10, 17–28), only the nuclear proteins poly(ADP-ribose) polymerase (PARP) (10), DNA-dependent protein kinase (DNA-PK) (25), the ribonucleoprotein U1–70 kDa (20), GTP dissociation inhibitor D4 (26), and huntingtin (48) have been shown to be cleaved by caspase-3 (CPP32/YAMA), one of the final known effectors of the protease cascade. It remains unknown whether the elimination of a particular protein or class of proteins essential for cell viability and integrity is the target of the caspase protease cascade, or the degradation of multiple substrates without a particular essential role, individually, could overcome the...

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1 The abbreviations used are: ICE, interleukin-1-converting enzyme; PIPES, 1,4-piperazinediethanesulfonic acid; PARP, poly(ADP-ribose) polymerase; DNA-PK, DNA-dependent protein kinase; DSE, differentiation-specific element; DSEB, differentiation-specific element-binding protein; PCNA, proliferating cell nuclear antigen; PAGE, polyacrylamide gel electrophoresis; RF-C, replication factor C.
DSEB/RF-C140

| DSEB/RF-C140 | PARP | Sequence |
|--------------|------|----------|
| 195-197      |      | EDEKKEKRTNYGAYRSLYNREGPKALQGSGKEIPK |
| 198-221      |      | EDKTELKKGGLPAILKEGKRGKDEVDGDTDEVAK |
| 222-235      |      | Identical 19/22 (89%) |
| 236-252      |      | Positive 23/28 (83%) |

DSEB/RF-C140

| DSEB/RF-C140 | PARP | Sequence |
|--------------|------|----------|
| 402-425      |      | SIERDEAKSLIERGGKVTVGNVSLQNLKDEAKSLIEK |
| 426-442      |      | LGGKLTGSANK |
| 443-454      |      | Identical 13/24 (54%) |
| 455-479      |      | Positive 20/24 (83%) |

DSEB/RF-C140

| DSEB/RF-C140 | PARP | Sequence |
|--------------|------|----------|
| 211-215      |      | DDEVG |
| 216-221      |      | DDEVG |
| 222-226      |      | Identical 5/5 (100%) |

FIG. 1. Sequence homologies between the amino acid sequences of mouse DSEB/RF-C140 and poly(ADP-ribose) polymerase. The upper sequences are within the DNA-binding domain. The middle sequences have no ascribed functions. The lower sequences are the substrate for cleavages by CPP32 (arrow).

capacity for cells to repair themselves (29).

The mammalian DNA replication factor complex C, also called activation complex A1, is a multimeric complex formed by five polypeptides with apparent molecular masses of 140, 40, 38, 37, and 36 kDa (30, 31). This complex is required for DNA replication in mammalian cells. It recognizes and binds the primed DNA template in the replication fork and recruits PCNA to allow the assembly of a replication complex with the DNA polymerases δ (delta) and ε (epsilon) (31, 32). We fortuitously cloned the large subunit of this complex (designated DSEB) while searching for a nuclear protein with capacity to bind a differentiation-specific element (DSE) in the angiotensinogen gene promoter (33). Binding of DSEB to the DSE enables irreversible induction of angiotensinogen expression during adipocyte differentiation (34). The large subunit of the replication factor C, DSEB/RF-C140 is responsible for recognition of the primed DNA template and the recruitment of the other four polypeptides of the RF-C complex to the replication fork (31, 32). A large carboxy-terminal region with extensive homology to the other subunits of the RF-C is required for their association (35). Domain B within the carboxy-terminal region is required for the direct interaction of DSEB/RF-C140 with proliferating cell nuclear antigen (PCNA) (36). We have mapped the DNA-binding domain of DSEB/RF-C140 to an amino-terminal region of 126 amino acids (residues 367–493) (33). This domain has extensive homology to the prokaryotic DNA ligases and the eukaryotic enzyme, poly(ADP-ribose) polymerase, involved in the recognition of DNA breaks and the orchestration of a DNA repair response (37, 38). In its domain B, DSEB/RF-C140 also shares with PARP a conserved region with the consensus sequence DEVDG that is the cleavage site for caspase-3 during apoptosis. This homology suggests that DSEB/RF-C140 could also be a substrate for caspase-3 (CPP32, YAMA). Such a cleavage would inactivate DSEB/RF-C140 by separating the DNA-binding domain from the association domain required for its interaction with the other subunits of the replication factor C (35), and would compromise its direct interaction with PCNA (36). Our results confirm the predictions and indicate that DSEB/RF-C140 is cleaved into two fragments during Fas-induced apoptosis in T cells. By using site-directed mutagenesis, we identified the Asp706 residue to be essential for the cleavage. This residue is contained in the DEVDG sequence with homology to the CPP32 cleavage site in PARP. Furthermore, by using specific protease inhibitors, we identified caspase-3 (CPP32) or a very close homolog protease, and not caspase-1 (ICE) as the cleaving protease. As in the case of PARP and other substrates, for the CED3-like proteases, the exact relevance of their cleavages in apoptosis remains to be determined. However, the identification of DSEB/RF-C140 as a substrate cleaved by caspase-3 or a closely related protease may be an important clue for understanding the role that ICE/CED3-like proteases play during programmed cell death.

EXPERIMENTAL PROCEDURES

Materials—DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim. Radioactive compounds were purchased from NEN Life Science Products. Nucleotides were obtained from Pharmacia Biotech Inc. Tissue culture media and reagents were from Life Technologies, Inc. Other reagents were purchased from Sigma. The cowpox-virus serpin CrmA was obtained from Kimm Remedi Biomedical Co. (Tukwila, WA). ICE and CPP32 specific tetrapeptide aldehyde antagonists were obtained from Peptide Institute Inc. (Osaka, Japan).

Cells and Culture Conditions—Jurkat cells were obtained from the ATCC (Rockville, MD) and grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). To induce apoptosis, 1 ml aliquots containing 2 × 10⁶ cells were exposed to the indicated concentrations (0.1–1 μg/ml) of the anti-human Fas monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) for the indicated periods of time.

Plasmid Construction and Mutagenesis—The 0.6-kilobase pair DNA containing the entire protein coding sequence for DSEB/RF-C140 was inserted into the pCDNA-1 vector described previously (33). By using site-directed mutagenesis, a mutated version of DSEB/RF-C140, DSEB Asp706 → Ala, was generated. In this mutated protein, the normal Asp residue at position 706 was converted to an alanine residue. Oligonucleotides ATGATGAGGTCGCCAGCGACCTCATCCATGAGGGCGTG and GCCTGCCATGCCAGCGACCTCATCCATGAGGGCGTG were used to create the mutation using a polymerase chain reaction-based technique.

In Vitro Transcription and Translation—Full-length RF-C140/DSEB protein was synthesized in reticulocyte extracts by in vitro transcription and translating the DNA sequence contained in the pCDNA-DSEB plasmid (33), using T7 RNA polymerase in the TNT system (Promega, Madison, WI). The incorporation of [35S]methionine (>1000 Ci/mmol) of the anti-human Fas monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) for the indicated periods of time

In Vitro Proteolytic Cleavage Assay—Incubation of in vitro translated DSEB/RF-C140 with a cytoplasmic extract from Fas antibody-treated or non-treated Jurkat cells, or a bacterial extract containing recombinant NCE or CPP-32, gift from Dr. J. Yuan (Harvard University, Boston, MA), was performed at 37°C during 1 h in a final 20-μl volume. After addition of Laemmli buffer, samples were loaded on a 10% SDS-PAGE, and the gel was dried and autoradiographed. Cytosolic extracts were prepared by two different methods. No difference between their cleavage capacity was observed. The first method was a modification of a previously described method (13). Briefly, after incubations, cells were washed twice in cold phosphate-buffered saline and resuspended in 100 μl of extraction buffer containing 50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonlfyl fluoride, then repeated cycles of freezing and thawing were applied. The second method used also has been described (39).
FIG. 2. *In vivo* cleavage of DSEB/RFC-140 during Fas-induced apoptosis in Jurkat T cells. 

A, Fas antibody dose-dependent DNA degradation. 

B, Fas antibody dose-dependent DSEB/RFC-140 cleavage detected by Western blot, using an antiserum directed against the amino-terminal region of DSEB. 

C, time-dependent DNA degradation. 

D, time-dependent DSEB/RFC-140 proteolytic cleavage. 

E, time-dependent cleavage of PARP, detected on the same filter as DSEB/RFC-140 after stripping and reprobing with a PARP monoclonal antibody.
RESULTS AND DISCUSSION

**DSEB/RF-C140 and PARP Are Structurally Related**—As a consequence of screening cDNA expression libraries for proteins that bind to a DSE in the angiotensinogen gene promoter (34), previously we cloned a nuclear DNA-binding protein, DSEB (differentiation specific element-binding protein), the expression of which is induced during the process of the differentiation of 3T3-L1 adipoblasts into adipocytes (33). Although initially no related sequences were available in the GenBank™ data base, an identical cDNA sequence was published that encoded the large subunit of the DNA replication factor C (41, 42). We have mapped the DNA-binding domain of DSEB to a region comprising residues 367–493 (33). This region is structurally related to two major families of proteins: bacterial DNA ligases and PARP (38). PARP is also a large nuclear protein, which is able to recognize and bind damaged DNA. PARP is important in the process of DNA repair because, once bound to DNA, it becomes active and orchestrates the repair process by ADP-ribosylating a number of nuclear proteins including itself. During the process of programmed cell death, PARP is inactivated due to specific cleavage of the protein into two fragments, one containing the DNA-binding domain and the other containing the catalytic domain. The active form of caspase-3 (CPP32/YAMA), an ICE/CED-3-like protease, is responsible for PARP cleavage through recognition of a specific site with the sequence DEVD/G (10). Fig. 1 shows the homology between the amino acid sequences of DSEB/RF-C140 and PARP. Significant regions of homology are found in the DNA-binding domain of DSEB/RF-C140 and two domains of PARP with no ascribed function. A small region in DSEB/RF-C140 with homology to the “DEAD box” family of RNA helicases, and residing in the recently mapped domain B required for PCNA interaction is identical to the consensus DEVD/G CPP32 cleavage site of PARP.

**In Vivo Cleavage of DSEB/RF-C140 during Fas-induced Apoptosis**—To determine whether DSEB/RF-C140 is also a substrate for ICE/CED-3 proteases during programmed cell death, we have used Jurkat cells treated with anti-Fas-reactive antibodies, a well established model for apoptosis. Jurkat T cells were incubated with anti-Fas-reactive monoclonal antibodies, and the appearance of apoptosis was measured by determining the pattern of migration of DNA in agarose-gel electrophoresis (DNA laddering) (Fig. 2A). Incubation of Jurkat cells with increasing concentrations of Fas antibodies causes a DNA laddering degradation pattern characteristic of apoptosis. Western immunoblot analyses were performed on the same cell extracts that were examined for DNA laddering. The antiserum used for the immunoblot analysis was raised against an amino-terminal fragment of DSEB/RF-C140 (33). The antiserum detects the DSEB/RF-C140 in control Jurkat cells and, upon the addition of Fas antibody to the cells, a new immunoreactive protein with a molecular mass of 87 kDa appears (Fig. 2B). The size of this new protein is consistent with a cleavage at the predicted DEVD/G site, between Asp' and Gly'. By using the lower effective dose (0.1 µg/ml) of Fas antibody, we determined whether the cleavage of DSEB/RF-C140 occurred earlier or later than the Fas-dependent apoptosis as measured by DNA degradation. Fig. 2C indicates that some DNA degradation is observed after 1 h of Fas antibody treatment and becomes intense after 2 h. A Western immunoblot prepared from cells incubated in parallel in the same experiment indicates that a marked cleavage of DSEB/RF-C140 occurs after 2 h of treatment with Fas antibodies (see Fig. 2D). After stripping and reprobing the same filter with a PARP monoclonal antibody (Fig. 2E), we observed that PARP cleavage occurs also in parallel with DNA degradation, starting 1 h after the addition of the Fas antibody. Although PARP cleavage seems to occur earlier than DSEB/RF-C140 cleavage, an increased concentration of PARP per cell or a more accessible pool could also explain an earlier detection. This finding indicates that the cleavage of DSEB/RF-C140 does not precede degradation of the DNA, but instead occurs in conjunction with DNA degradation.
and the reported cleavage of the other known CPP32 substrates (25, 28).

**In Vitro Proteolysis Assays Confirm That Fas-dependent Cleavage Occurs at Asp706**—An *in vitro* proteolysis system was established to study the cleavage pattern in DSEB/RF-C140 induced by cytosolic extracts from Fas-treated Jurkat T cells. DSEB/RF-C140 synthesized by coupled cell-free transcription and translation in a reticulocyte lysate was isolated by immunoprecipitation and incubated with a cytosolic extract from control or Fas-stimulated cells. In the presence of Fas-treated but not untreated cytosolic extracts, DSEB/RF-C140 was cleaved into two smaller fragments (Fig. 3), the large fragment corresponding in molecular weight to the amino-terminal fragment observed in the Western immunoblot studies with an antibody that recognizes the amino-terminal domain of DSEB/RF-C140. A new polypeptide with an estimated molecular size of 53 kDa was also observed. Depending on the electrophoresis conditions, the smaller protein fragment migrates as a duplex, perhaps indicating the existence of two different conformations or a secondary cleavage event. Although both the size of the cleaved fragments and the immunoreactive pattern indicate that the predicted CPP32 consensus, DEVGD(703–707) is the cleavage site during Fas-dependent apoptosis, we confirmed the possibility by replacing the Asp706 residue with an alanine by site-directed mutagenesis. As was anticipated, this new mutant protein was not cleaved by a Fas antibody-treated extract (Fig. 3). A similar Asp to Ala substitution in another CPP32 substrate, steroid response element-binding protein (SREBP), was also shown to prevent cleavage by CPP32 (27).

**DSEB/RF-C140 Cleavage Induced by Fas Treatment Is Due to Caspase-3 (CPP32) or a Closely Related Protease**—We further characterized the proteolytic activity responsible for DSEB/RF-C140 cleavage in Fas-treated Jurkat cells. Two different classes of proteases have been determined to be activated during Fas-dependent apoptosis in T cells: 1) a very early initial ICE-like activity, inhibited specifically by the tetrapeptide aldehyde Ac-YVAD-CHO; and 2) a later appearance of a CPP32-like activity, inhibited by the tetrapeptide aldehyde Ac-DEVD-CHO. By using the *in vitro* cleavage assay, we examined the capacity of these different compounds to inhibit DSEB/RF-C140 cleavage activity in cytosolic extracts prepared from Jurkat cells treated with Fas antibodies. Fig. 4A shows

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**Fig. 4.** The use of protease inhibitors specific for CPP32 and ICE indicates that CPP32 or a very closely related protease is responsible for DSEB/RF-C140 cleavage. A, effects of iodoacetamide and the specific tetrapeptide aldehyde inhibitors. B, effects of the cowpox-virus serpin CrmA. C, direct evidence that only CPP-32 and not ICE cleaves DSEB/RF-C140 is provided by using preparations of recombinant CPP-32 and ICE proteins.
that only the nonspecific cysteine-protease inhibitor iodoacetamide and the CPP32-specific inhibitor Ac-DEVD-CHO prevent the cleavage of DSEB/RF-C140 by the cytosolic extracts prepared from cells undergoing apoptosis. A non-cysteine-protease inhibitor such as leupeptin and the ICE-specific inhibitor AC-YVAD-CHO did not prevent cleavage. These experiments indicate that CPP32, or a very close homolog of CPP32, which shares its pattern of response to certain protease inhibitors, is responsible for the cleavage of DSEB/RF-C140. The cowpox virus CrmA serpin, a potent inhibitor of ICE without a significant effect on caspase-3 activity, does not prevent DSEB/RF-C140 cleavage, further indicating that ICE is not the protease responsible for DSEB/RF-C140 cleavage in cytosolic extracts from Fas antibody-treated cells (Fig. 4B). It has been shown that activation of ICE-like proteolytic activity is a very early event during Fas-induced apoptosis, whereas activation of CPP32 occurs later in parallel with the degradation of DNA (13). These data, reported earlier, are in agreement with our observations.

Specific DSEB/RF-C140 Cleavage by Recombinant Caspase-3 (CPP-32)—Further confirmation that only caspase-3 (CPP-32) and not caspase-1 (ICE) is the proteolytic activity responsible for DSEB/RF-C140 cleavage was obtained by using a bacterial preparation of recombinant proteases (gift from Dr. J. Yuan). Fig. 4C shows that when "in vitro" translated DSEB/RF-C140 is incubated in the presence of recombinant CPP-32, cleavage occurs. This cleavage has the same pattern as the one induced by cytoplasmic extracts from Fas-treated Jurkat cells. On the other hand, recombinant ICE (caspase-1) has no cleavage activity on DSEB/RF-C140 as is predicted by the studies using specific inhibitors.

DSEB/RF-C140 Cleavage Inactivates Its Functional Role in DNA Replication—Caspase-3 cleavage of DSEB/RF-C140 at Asp706 during apoptosis results in disappearance of the normal full-length protein and the generation of two new fragments. These two newly formed proteins still contain clusters of functional domains of the wild-type DSEB/RF-C140 that theoretically could support some DNA replication activity. However, during the preparation of this manuscript, an extensive study of the replication function of the RF-C140 was published (50). Because the reported deletion mutants deficient in DNA replication comprise the same clusters of functional domains as the two caspase-3 generated fragments, it is concluded that both fragments are inactive and do not support DNA replication.

An Emerging Common Pattern of CPP32 Substrate Cleavage—Activation of CPP32, as defined by inhibition by the tetrapeptide Ac-DEVD-CHO, is an early event during programmed cell death and its exact role in apoptosis is unknown. Activated CPP32 has been shown to cleave only a selected number of nuclear substrates: PARP (10), DNA-PK (25, 28), and the U1–70 kDa subunit of the small nuclear ribonucleoprotein complex (20). Our study adds a fourth protein, DSEB/RF-C140, to this relatively short list of specific nuclear substrates. In all these instances, cleavage occurs between an anchoring domain (DNA- or RNA-binding domains) and a functional or catalytic domain (Fig. 6). Cleavage of these substrates occurs in parallel with nucleosomal DNA degradation. This circumstance may indicate that cleavage of substrates and DNA degradation are not causally linked and that they occur independently from one another. This concept has recently received experimental support (49). Because both PARP and DNA-PK are involved in DNA repair processes, it has been proposed that their cleavages, if not essential, at least would facilitate the completion of the apoptotic process by blocking the DNA repair capacity of those cells committed to the programmed cell death (44). Because RF-C is required for PCNA loading onto DNA, and PCNA is not only required for DNA replication but also for the DNA excision-repair mechanisms (45), it has been suggested that the RF-C could also participate in certain types of DNA repair processes where PCNA is involved. If this were the case, cleavage of the DSEB/RF-C140 by
disruption of its B domain required for PCNA interaction (36) would almost certainly impair its role in DNA repair, contributing to the completion of DNA degradation. However, another and perhaps more interesting possibility is that suppression of DNA replication, especially the elongation phase of DNA replication performed by polymerase δ and ε, is a necessary or facilitating step for the completion of DNA degradation and the apoptotic process. It seems unquestioned that both suppression of DNA synthesis, as well as suppression of DNA repair mechanisms are logical events that might occur in a cell that is degrading its DNA as part of its commitment to a predetermined and organized suicide program. Another feature that the known CPP32 substrates share among themselves is that they are abundant ubiquitously distributed proteins (25). DSEB/RF-C140 itself is present in at least 10,000 molecules/cell (42). They also share the capacity to consume ATP as a source of energy. Therefore, the inactivation of the CPP32 substrate is expected that the rate of DNA degradation would increase, resulting in a shortening of the critical time required for the completion of the apoptotic process.

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