Apoptosis (programmed cell death) is a fundamental process for normal development of multicellular organisms, and is involved in the regulation of the immune system, normal morphogenesis, and maintenance of homeostasis. ICE/CED-3 family cysteine proteases have been implicated directly in apoptosis, but relatively few substrates have been identified. Here we report that D4-GDI, an abundant hematopoietic cell GDP dissociation inhibitor for the Ras-related Rho family GTPases, is a substrate of the apoptosis protease CPP32/Yama/Apopain. D4-GDI was rapidly truncated to a 23-kDa fragment in Jurkat cells with kinetics that parallel the onset of apoptosis following Fas cross-linking with agonistic antibody or treatment with staurosporine. Fas- and staurosporine-induced apoptosis as well as cleavage of D4-GDI were inhibited by the ICE inhibitor, YVAD-cmk. D4-GDI was cleaved in vitro by recombinant CPP32 expressed in Escherichia coli to form a 23-kDa fragment. The CPP32-mediated cleavage of D4-GDI was completely inhibited by 1 μM DEVD-CHO, a reported selective inhibitor of CPP32. In contrast, the ICE-selective inhibitors, YVAD-CHO or YVAD-cmk, did not inhibit CPP32-mediated D4-GDI cleavage at concentrations up to 50 μM. N-terminal sequencing of the 23-kDa D4-GDI fragment demonstrated that D4-GDI was cleaved between Asp19 and Ser20 of the poly(ADP-ribose) polymerase-like cleavage sequence DELD19S. These data suggest that regulation by D4-GDI of Rho family GTPases may be disrupted during apoptosis by CPP32-mediated cleavage of the GDI protein.

Apoptosis (programmed cell death) acts to preserve peripheral T cell homeostasis, participating in the elimination of both immature thymocytes during thymic development and mature peripheral T cells following antigen stimulation under certain conditions (1–3). Fas (CD95), a member of the TNF receptor/nerve growth factor family (4), is highly expressed in activated lymphocytes (5), and the ligand for Fas (FasL) appears to be expressed exclusively on activated T cells (6, 7). Fas-mediated apoptosis is involved in down-regulation of immune reactions as well as in T cell-mediated cytotoxicity (8). Genetic mutations in murine Fas (lpr mutation) or FasL (gld mutation) lead to defective T cell receptor-induced cell death of mature T cells, resulting in autoimmune disease (10). A human dominant interfering Fas mutation has also been described that leads to autoimmune lymphoproliferative syndrome (11). More recently, Fas-induced apoptosis has been implicated in establishing immune-privileged sites such as the testes and eye, HIV elimination of T cells, and cytotoxic T lymphocyte-mediated cell killing (8, 12–17).

ICE/CED-3 family cysteine proteases have been implicated directly in apoptosis as evidenced by the findings that overexpression of ICE-like proteases results in apoptosis and that co-expression of the viral proteins, CrmA and P35, which inhibit ICE family proteases can prevent the associated cell death (13, 18–23). Deletion of ICE in mice renders thymocytes resistant to apoptosis induced by Fas, but not by dexamethasone or γ-irradiation (24, 25). A related protease, CPP32/Yama/Apopain (18, 26, 27), specifically cleaves the nuclear protein poly(ADP-ribose) polymerase after induction of apoptosis, and inhibition of CPP32 activity by either a peptide inhibitor or by CrmA attenuates apoptosis in vitro (18, 26). Furthermore, CPP32 is also involved in cytotoxic T lymphocyte-mediated target cell lysis following its activation through cleavage by granzyme B (28). These data implicate CPP32 as an important ICE/CED-3 family protease directly involved in the initiation of apoptosis.

Relatively few apoptosis-related substrates for the ICE/CED-3 family proteases have been reported, and the role of these substrates in apoptosis remains unclear. Due to their location and function, the ICE/CED-3 family protease substrates poly(ADP-ribose) polymerase and lamin are potentially important in the characteristic nuclear changes associated with apoptosis (18, 26, 30). The cellular signaling pathways involved in controlling apoptosis remain poorly defined as well. In particular, little is known about the mechanisms underlying the dramatic cytoskeletal, morphological, and membrane changes that accompany cell death and that may be important in the subsequent recognition and disposal of apoptotic cells by phagocytic leukocytes. In normally growing cells, such processes have been shown to be controlled by the action of Rho family GTPases (31, 32). We establish here that D4-GDI, a hematopoietic cell-abundant regulator of the Rho family GTPases (33, 34), is a substrate for CPP32 and is cleaved during apoptosis. These data suggest the likelihood of impor...
CPP32-mediated Cleavage of D4-GDI

D4-GDI is a highly abundant regulator of Rho GTPases in lymphoid and myeloid cells and is highly homologous to Rho-GDI, differing primarily at the N-terminal 25 amino acids (33, 34). During the purification of ICE activity from THP-1 cells, we had observed two truncated forms of D4-GDI co-purifying with ICE activity. Briefly, ICE was partially purified from THP-1 cell lysates by three steps of ion exchange chromatography. At this stage, ICE activity was purified about 3500-fold relative to cell lysate, and two protein bands could be seen co-migrating with ICE activity. N-terminal sequencing revealed that a band at 22 kDa was the p20 subunit of ICE, while a 19-kDa band was a truncated form of D4-GDI beginning at residue Gly62. Fig. 4, lane 2, shows a western blot analysis of the partially purified ICE fraction using antipeptide antibody raised against the N terminus of the truncated D4-GDI. In addition to the 19-kDa fragment, the antibody detected a 23-kDa fragment of D4-GDI. Inspection of the protein sequence revealed that the N-terminal region of D4-GDI contains two potential ICE/CED-3 protease cleavage sites with the sequences DELD135G and LLGIDSG (Fig. 1). Neither site is present in Rho-GDI. These led us to test whether D4-GDI was a specific substrate for ICE-like proteases during the process of Fas-induced T cell apoptosis.

CPP32-mediated Cleavage of D4-GDI

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induced cleavage of D4-GDI to the 23-kDa fragment (Fig. 3d).
The same treatment of Jurkat cells with up to 200 μM of a	nonspecific control inhibitor, Ac-AAPV-cmk, did not result in
significant inhibition of Fas- or staurosporine-induced apopto-
sis or D4-GDI cleavage (data not shown). These results suggest
that the inhibition of both apoptosis and D4-GDI cleavage by
Ac-YVAD-cmk is due to the specific inhibition of ICE or ICE-
related proteases.

Since D4-GDI appeared to be cleaved at a CPP32-like con-
sensus sequence (DELD19S), we tested whether recombinant
CPP32 would give appropriate cleavage of purified recombi-
nant D4-GDI as seen in the Jurkat T cells during apoptosis.
CPP32, expressed in E. coli as a GST-fusion protein, was au-
tomatically processed to its mature form as monitored by the cleavage of
the GST tag from the fusion protein with detection by Western
analysis using anti-GST antibody (data not shown). Cleavage
of D4-GDI by recombinant CPP32 was assessed by Western
analysis using anti-D4-GDI (Fig. 4). Incubation of purified re-
combinant D4-GDI with the E. coli extract containing CPP32
resulted in the cleavage of mature 28-kDa D4-GDI to a 23-kDa
fragment, identical in size to the fragment observed during
Fas-induced apoptosis of Jurkat T cells (Fig. 4, compare lanes 1
and 3). Recombinant Mch2 and ICErel-II, two additional ICE/
CED-3 family members, did not cleave D4-GDI under the same
conditions, nor did control E. coli extracts expressing only the
GST gene (data not shown). To confirm further the specific
cleavage of D4-GDI by CPP32, we examined three peptide
inhibitors for their ability to inhibit this cleavage. The tet-
rapeptide aldehyde Ac-DEVD-CHO, a potent inhibitor for
CPP32 (26), showed substantial inhibition of D4-GDI cleavage
at concentrations as low as 0.1 μM (Fig. 4, lanes 4–6). In
contrast, the ICE-specific tetrapeptide inhibitors, Ac-YVAD-
CHO and Ac-YVAD-cmk (16, 39), were less effective at inhib-
iting CPP32 activity for D4-GDI cleavage (Fig. 4, lanes 7–14).
Together, these results demonstrated that CPP32 is capable of
specifically cleaving D4-GDI to produce a 23-kDa fragment
that is identical in size to the fragment generated during Fas or
staurosporine-induced apoptosis.

A significant difference in the sensitivity to inhibition by
Ac-YVAD-cmk on CPP32-mediated D4-GDI cleavage in vitro
versus Fas-induced Jurkat cell apoptosis was observed in these
experiments. 5 μM Ac-YVAD-cmk blocked approximately 90% cell
death and D4-GDI cleavage during Fas-induced apoptosis
in Jurkat cells (Fig. 3, a and c). In contrast, CPP32-mediated
cleavage of D4-GDI in vitro was only minimally inhibited by
Ac-YVAD-cmk at a concentration of 10 μM (Fig. 4, lane 13). This
difference in sensitivity may be attributed to inhibition of an
ICE-related enzyme that is required for processing and activa-
tion of CPP32 following Fas induction. This possibility is sup-
ported by the findings that transgenic deletion of ICE in mice
abrogated Fas-mediated apoptosis of thymocytes (24) and that
CPP32 can be processed in vitro by ICE (18). In contrast to
Fas-induced apoptosis, 50 μM or higher concentrations of Ac-
YVAD-cmk were required to block staurosporine-induced ap-
optosis and associated D4-GDI cleavage in Jurkat cells. These
concentrations were more in line with in vitro inhibition of
CPP32-mediated D4-GDI cleavage (Fig. 3 versus Fig. 4). These
results suggest that staurosporine-induced apoptosis proceeds
through a distinct pathway that is independent of Ac-YVAD-cmk inhibitable protease(s).

In order to confirm the exact CPP32 cleavage site of D4-GDI, the essential Asp residues at the respective P1 positions (26, 39) of both putative ICE-protease cleavage sites were mutated (see Fig. 1). Both wild type and mutant proteins were translated as 35S-Met labeled protein in an in vitro transcription/translation system. Recombinant CPP32 was added to the lysates containing either wild type or mutant D4-GDI, and the cleavage of

Fig. 3. Dose-dependent inhibition of anti-Fas antibody- and staurosporine-induced apoptosis and D4-GDI cleavage in J urkat cells by the tetrapeptide inhibitor, Ac-YVAD-cmk. J urkat cells were preincubated for 3 h with various concentrations of Ac-YVAD-cmk (Bachem) and then stimulated with anti-Fas antibody (CH11) (a) or 1 μM staurosporine (c) for an additional 3 h at 37 °C. Apoptotic cells were measured as described under "Materials and Methods." For the determination of D4-GDI cleavage, cell lysates prepared from anti-Fas antibody- (b) or staurosporine-treated (d) cells were analyzed by Western blotting with D4-GDI antibody as described. The D4 antibody-reactive bands observed were as described in the Fig. 2 legend. The data shown are the average of two independent experiments.
CPP32-mediated Cleavage of D4-GDI

23-kDa cleavage product observed during apoptosis in Jurkat cells.

D4-GDI was first identified as a hematopoietic cell-specific homolog of Rho-GDI, a negative regulator of Rho family GTPases (33, 34). These proteins form a complex with members of the Rho GTPase family (Rho, Rac, and Cdc42) and thereby maintain an inactive, cytosolic form of the GTPase (40). The cellular signals that cause disruption of the complex, thereby resulting in conversion to the active, GTP-bound form of the GTPase through the action of guanine nucleotide exchange factors (41), are not known, although biologically active lipids can display this activity in vitro (40). Here we demonstrate that D4-GDI is specifically cleaved by CPP32 during Fas-induced apoptosis, suggesting a novel means of regulation of such complexes. Cleavage through the action of CPP32 produces an irreversible modification specifically of D4-GDI (versus Rho-GDI), removing the most divergent portion of the protein. Indeed, the initial 20 amino acids of Rho-GDI appear to be critical determinant of whether cells undergo an apoptotic response (29). We will direct future studies toward understanding the relevance of D4-GDI cleavage by CPP32 to the dramatic membrane, cytoskeletal, and biochemical changes that accompany the apoptotic process.

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