Caspases are activated during apoptosis and cleave specific proteins, resulting in the irreversible commitment to cell death. The signal transduction proteins MEKK1, p21-activated kinase 2, and focal adhesion kinase are caspase substrates that contribute to the cell death response when cleaved. Thirty additional signaling proteins were screened for their ability to be cleaved during apoptosis. Twenty-two of these proteins were not affected in Jurkat cells stimulated to undergo apoptosis by Fas ligation, exposure to ultraviolet-C or incubation with etoposide. Ras GTPase-activating protein was found to be a caspase substrate whose cleavage followed the same time course as that for activation of caspase activity and the cleavage of MEKK1 and focal adhesion kinase. Four additional proteins, Cbl, Cbl-b, Raf-1, and Akt-1, were cleaved later in the apoptotic response. These signaling proteins were similarly cleaved in U937 cells undergoing apoptosis. Cleavage of the proteins was blocked by caspase inhibitors in Jurkat cells or in U937 cells expressing BclxL, demonstrating that the cleavage was dependent on caspase activation. Cleavage of Raf-1 and Akt correlated with the loss of extracellular signal-regulated kinase and Akt activities in apoptotic cells. Neither c-Jun N-terminal kinase nor p38 mitogen-activated protein kinase protein was cleaved in cells undergoing apoptosis, and the activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways was not compromised in apoptotic cells. These results indicate that caspase-dependent cleavage of specific proteins induces the turn off of survival pathways, such as the extracellular signal-regulated kinase and phosphatidylinositol-3 kinase/Akt pathways, that could otherwise interfere with the apoptotic response.

Apoptosis is regulated by a series of biochemical events that commit a cell to death. A common feature of cells undergoing apoptosis is the activation of caspases, a family of aspartic acid-directed proteases (1). Caspase substrates are rapidly being identified, but the general assumption is that caspases recognize a limited set of cellular proteins (2). Caspase-mediated proteolysis of specific proteins results in an irreversible commitment of cells to undergo apoptosis characterized by cytoplasmic shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation.

Caspase-dependent cleavage can inactivate protein substrates. Examples include poly(ADP-ribose) polymerase, lamin, and focal adhesion kinase. Cleavage of poly(ADP-ribose) polymerase abolishes its DNA repair ability in cells undergoing apoptosis, nuclear lamin degradation contributes to nuclear condensation, and cleavage of focal adhesion kinase impairs the ability of cells to maintain matrix adherence (2). In contrast, there are examples, besides the caspases themselves (3), where cleavage actually activates the substrate; examples of such substrates include MEKK1, p21-activated kinase, protein kinase Cδ, and gelsolin. When cleaved, each of these proteins contributes to the apoptotic response (4–9).

Signal transduction pathways involving the mitogen-activated protein kinases (MAPKs) including the ERKs, JNKs, and p38/HOG1 kinase, have been shown to differentially contribute to pro- and anti-apoptotic pathways (10). In addition, the phosphatidylinositol 3-phosphate-regulated protein kinase Akt has been shown to have significant anti-apoptotic signaling properties (11). This is, at least in part, mediated by the ability of Akt to phosphorylate and inactivate BAD, a pro-apoptotic member of the Bcl family (12, 13).

In this study, we have surveyed a large set of proteins that are involved in pathways regulating cell growth, cell survival, or cell death, including members of the MAPK network. Among 30 signaling proteins tested, we have identified five new protease substrates, RasGAP, Raf1, Akt-1, Cbl, and Cbl-b, that were found to be cleaved in a caspase-dependent manner during the apoptotic response induced by Fas activation, ultraviolet-C (UV-C) irradiation, and etoposide. Cleavage of Raf-1 and Akt-1 inhibited their kinase activity. This could explain why the anti-apoptotic ERK and Akt pathways are inhibited during the progression of apoptosis.

EXPERIMENTAL PROCEDURES

Cells—Jurkat and U937 cells were cultured in RPMI 1640 (Life Technologies, Inc.; catalog number 31800-022), supplemented with 100 units/ml penicillin/streptomycin (Gemini Bio-Products) and containing 10% fetal calf serum (Summit Biotechnology) (RPMI-c).

Fas Stimulation—Jurkat cells were incubated with 1 μg/ml anti-Fas IgM antibodies (Upstate Biotechnology, Inc.; catalog number 05-201) in phosphate-buffered saline for 20–30 min on ice. The cells were then washed twice with phosphate-buffered saline, resuspended in RPMI-c, and incubated for the indicated periods of time at 37 °C, 5% CO2. When caspase inhibitors were used, they were incubated with Jurkat cells

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both during the incubation with anti-Fas antibodies and during the incubation at 37 °C.

In Vitro Kinase Assays—The cells were solubilized in TX-100 lysis buffer (70 mM β-glycerophosphate, 1 mM EGTA, 100 μM Na3VO4, 1 mM dithiothreitol, 2 mM MgCl2, 0.5% Triton X-100, 20 μg/ml aprotinin). Cell-associated kinase activity was removed by centrifugation at 800 × g for 5 min. Protein concentration was determined by a Bradford assay using bovine serum albumin as a standard.

c-Jun Kinase—c-Jun kinase (JNK) activity was measured using a solid phase kinase assay in which glutathione S-transferase-c-Jun(1–79) (GST-Jun) bound to glutathione-Sepharose 4B beads was used to affinity-purify JNK from cell lysates as described (14, 15). Quantitation of the phosphorylation of GST-Jun was performed with a PhosphoImager (Molecular Dynamics).

ERK—ERK2 was incubated with 2 μg/ml of an anti-ERK2 (C-14) antibody (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C with agitation, followed by the addition of 15 μl of a 1:1 slurry of protein A-Sepharose beads (Sigma, catalog number P-3391) and a further 20-min incubation at 4 °C. The beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of lysis buffer without Triton X-100. Thirty-five μl of the last wash was left in the tube and mixed with 20 μl of ERK reaction mix (50 mM β-glycerophosphate, 100 μM Na3VO4, 10 mM MgCl2, 200 μM ATP, 0.5 μCi/μl γ-32P]ATP, 400 μM epidermal growth factor receptor peptide 662–681, 100 μg/μl IP-20, 2 mM EGTA), incubated for 20 min at 30 °C. The reaction was stopped with 10 μl of 25% trichloroacetic acid and spotted on P81 Whatman paper. The samples were washed three times with 5 ml each in 75 mM phosphoric acid and once for 2 min in acetone and air-dried, and their radioactivity was determined in a β-counter.

Akt1—Four hundred μg of cell lysates was immunoprecipitated with 2 μg/ml of an anti-Akt1 antibody (Santa Cruz Biotechnology, Inc.; catalog number sc-1618) as described for the ERK assay. The beads were then washed twice in 1 ml of lysis buffer and twice in 1 ml of Akt wash buffer (20 mM Tris, pH 7.5, 10 mM MgCl2, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1 μg/ml IP-20). Thirty-five μl of the last wash was left in the tube and mixed with 25 μl of Akt reaction mix (Akt wash buffer, 0.2 mM ATP, 0.2 mM/mg cross-tide peptide (GRPRTRSSSFAEG), 0.2 μCi/μl γ-32P]ATP) and incubated for 20 min at 30 °C. The reaction was stopped with 10 μl of 0.5% EDTA and spotted on P81 Whatman paper as described for the ERK assay.

p38–p38 activity was measured exactly as described by Gerwins et al. (16).

Immunoblots—200–400 μg of cell lysate protein was subjected to SDS–7–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were performed exactly as described (17).

Measurements of Caspase Activities—Cells were lysed in 50 mM Tris, pH 7.4, 1 mM EDTA, 10 mM EGTA. Sixty μg of lysate proteins were incubated with 5 μM DEVD-7-amido-4-methylcoumarin (Bachem) in 1 ml of 50 mM Tris, pH 7.4, 1 mM EDTA, 10 mM EGTA for 20 min at 37 °C. Fluorescence was then monitored with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Fluorescence of the substrate alone was subtracted in each case.

Measurement of Apoptosis—Cells (1–2 × 106) were resuspended in 100 μl of incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2) containing 1 μg/ml propidium iodide (Sigma; P-4170) and a 1:50 dilution of Annexin-V-Flos solution (Boehringer Mannheim; catalog number 1828681) and incubated 15 min on ice. Four hundred μl of incubation buffer was then added, and the cells were sorted on a flow cytometer using 488-nm excitation and a 515-nm bandpass filter for fluorescence detection and a filter >560 nm for propidium iodide detection. Apoptotic cells were defined as green fluorescent positive and propidium iodide negative.

Plasmids—Akt1.dn3 corresponds to the mouse Akt-1 cDNA (nucleotides 254–1729) subcloned into the pcDNA3 eukaryotic expression vector (InVitrogen). h_Cbl.dn3 corresponds to the human Cbl cDNA (nucleotides 254–1729) subcloned into the pcDNA3 eukaryotic expression vector (InVitrogen). h_Cbl.dn3 corresponds to the human Cbl cDNA (nucleotides 254–1729) subcloned into the pcDNA3 eukaryotic expression vector (InVitrogen). h_Cbl.dn3 corresponds to the human Cbl cDNA (nucleotides 254–1729) subcloned into the pcDNA3 eukaryotic expression vector (InVitrogen). h_RasGAP.dn3 corresponds to the human RasGAP cDNA (nucleotides 133–2977) in pcDNA3. SEK1.rst corresponds to the mouse SEK1 cDNA (nucleotides 16–2292) in pRSETb, and MEKK1.dn3 corresponds to the mouse MEKK1 cDNA in pcDNA3.

In Vitro Translocation—Proteins were in vitro translated using the TNT® T7 coupled reticulocyte lysate system (Promega) as per the manufacturer's conditions. The plasmids used were Akt1.dn3, h_Cbl.dn3, h_RasGAP.dn3, JNK1.rst, MEK1.rst, Raf1.rst, h_RasGAP.dn3, SEK1.rst, and MEKK1.dn3. The cleavage assay of the in vitro translated proteins was performed in the buffer used to measure DEVD-directed caspase activity (see above).

RESULTS

The fate of specific cellular proteins involved in the regulation of signal transduction during the apoptotic response was analyzed. Jurkat cells were treated with etoposide, a topoisomerase inhibitor; UV-C (254-nm) irradiation, which generates oxygen radicals and induces RNA/DNA damage; or anti-Fas antibodies that activate Fas. The apoptotic response was temporally defined for each stimulus by measuring annexin V positive cells and the activation of DEVD-directed caspases (Fig. 1). Both annexin V binding and caspase activation are well defined markers of apoptosis (18). In addition, Jurkat cells undergoing cell death showed morphological changes characteristic of apoptosis, including cytoplasmic shrinkage and nuclear condensation (data not shown). Fas ligation induced a rapid apoptosis as assessed by the appearance of annexin V binding at the cell surface after 15 min that reached a maximum after 1 h. UV-C (100 J/m2) irradiation and incubation with etoposide (30 μM) induced a slower cell death response that reached a maximum for both stimuli at approximately 8 h. The kinetics of DEVD-caspase activation paralleled the induction of annexin V binding to the cells.

Immunoblot analysis was performed against lysates from cells treated with etoposide, UV-C, or Fas antibody for different times with antibodies directed against 35 proteins having functions involved in different signaling pathways (Fig. 2 and Table 1). Of these proteins, the expression of 22 was not significantly altered during apoptosis. These included ERK1, ERK2, and the p85 and p110 subunits of PI3K that have been implicated in survival responses in different cell types (11, 14, 19) as well as components of the pro-apoptotic p38 and JNK MAPK pathways (JNK-1, SEK-1, p38 MAPK). Four proteins, phospholipase
Caspase-induced Cleavage of Signaling Proteins

Caspase-induced Cleavage of Signaling Proteins in apoptotic Jurkat cells. Jurkat cells, treated as in Fig. 1, were lysed and analyzed by Western blot for the presence of the indicated proteins using the antibodies described in Table I. TRAF-1, tumor necrosis factor receptor-associated protein-1, PLCγ1, phospholipase C-γ1.

Nine proteins showed essentially complete proteolysis during the apoptotic response. Proteolysis of these proteins occurred somewhat late in the apoptotic response. The fragments were clearly visible by immunoblotting, but the cleavage was incomplete and did not appear to significantly affect the amount of the full-length proteins.

Nine proteins showed essentially complete proteolysis during the apoptotic response. These proteins could be categorized into two groups based on their rate of cleavage following an apoptotic response. The first group is characterized by rapid cleavage after an apoptotic stimulus (extensive cleavage in 1 h of Fas stimulation). The prototypes of rapidly cleaved proteins are the caspases themselves, which are synthesized as inactive zymogens and are cleaved during their activation (3). The kinases ICH1 (caspase-2) and CPP32 (caspase-3) cleavage are shown in Fig. 2. Of the proteins assayed, three major signal transduction proteins were cleaved as fast as ICH1 and CPP32 (Fig. 2). These included the previously characterized caspase substrates MEKK1 and focal adhesion kinase (5, 6, 20).

Surprisingly, we found that the 120-kDa RasGAP protein was also rapidly cleaved with a similar time course as that for caspase activation. The second group of signaling proteins were cleaved with slower kinetics in Fas-stimulated cells (extensive cleavage detected only after 2 h of Fas stimulation). These were the adapter proteins Cbl and Cbl-b and the serine-threonine kinases Raf-1 and Akt-1. Etoposide and UV-C, which are slow stimulators of apoptosis compared with anti-Fas antibodies (Fig. 1), induced the cleavage of the proteins of both groups between 8 and 16 h and between 4 and 8 h of treatment, respectively (Fig. 2). Because of their involvement in the PI3K/Akt and the ERK MAPK pathways, shown to have survival functions in different cell types (11, 14, 19), Cbl and Cbl-b (which can bind PI3K), Raf-1 (the upstream regulator of the ERKs), and Akt-1 each have potential anti-apoptotic functions.

The cleavage of RasGAP, Raf-1, Cbl/Cbl-b, and Akt-1 in response to apoptotic stimuli has not been described previously. To demonstrate the generality of these signaling proteins as substrates for proteolysis during apoptosis, their cleavage in U937 cells exposed to UV-C was examined (Fig. 3). RasGAP is cleaved with a similar time course as that for CPP32. Cbl/Cbl-b, Raf-1, and Akt-1 were also cleaved but with slower kinetics. As controls, the dual specificity phosphatase, MAPK phosphatase-1, and the p110 subunit of PI3K were not degraded during the U937 cell apoptotic response.

The cleavage of proteins during the apoptotic response is highly restricted and is not a general degradation of cellular proteins. Using mild (0.5% TX-100-containing buffer) to strong (2% SDS-containing buffer) extraction procedures, no difference in Coomassie-stained protein profiles is observed between lysates from control and apoptotic Jurkat cells (Fig. 4A). However, selective cleavage of proteins did occur as shown in the immunoblots in Fig. 4B (RasGAP is cleaved, while CD45 or phospholipase C-γ1 are unaffected).

To define whether the cleavage of RasGAP, Cbl/Cbl-b, Raf-1, and Akt-1 resulted from caspase activation, Jurkat cells were incubated with or without cell-permeable caspase inhibitors during Fas ligation-induced apoptosis (Fig. 5A). The caspase inhibitor effectively blocked the cleavage of each of the proteins tested, including MEKK1, a defined caspase substrate (4, 6). Similarly, U937 cells overexpressing BclxL, which has anti-apoptotic functions and is involved in the regulation of caspase activation (21, 22), also blocked cleavage of RasGAP, Akt-1, Cbl, Raf-1, and CPP32 in response to UV-C irradiation (Fig. 5B). Thus, the activation of caspases is required for the cleavage and degradation of these proteins in both Jurkat and U937 cells.

Lysates from Fas-activated Jurkat cells have proven to be a good system for the assay of DEVD-directed caspases (4, 18). Therefore, lysates were prepared from control and Fas-ligated T cells to which [35S]methionine labeled in vitro translation products for MEKK1, RasGAP, Akt-1, Cbl, Raf-1, and three control proteins shown not to be degraded during apoptosis, SEK1, MEK1, and JNK1. Only MEKK1 and RasGAP were shown to be proteolyzed when added to the lysate from Fas-activated cells (Fig. 6). Purified CPP32 generated the same RasGAP fragments as those generated by lysates from apoptotic Jurkat cells (data not shown). This defines RasGAP as a DEVD-directed caspase substrate, as is MEKK1. Cbl, Akt-1, and Raf-1 were not proteolyzed in lysates from Fas-activated Jurkat cells, indicating that they are not cleaved by the caspases proteolyzing RasGAP and MEKK1 in the in vitro conditions used here. Their cleavage is, however, dependent on the activation of caspases as demonstrated in Fig. 5.

To assess the significance of protein cleavage on signaling pathways, we measured the activity of the ERK, JNK, and p38 MAPK pathways in Fas-stimulated Jurkat cells (Fig. 7A). The ERK pathway was transiently stimulated by anti-Fas antibodies as reported previously (23), reaching a maximum 1 h following Fas stimulation and returning to basal levels 2 h fol-
Caspase-induced Cleavage of Signaling Proteins

### Table I

**Antibodies used in this study**

| Antigen | Antibody (dilution/concentration) | Company or reference | Catalog number |
|---------|----------------------------------|----------------------|----------------|
| Human 14–3-3β | Rabbit IgG (1 µg/ml) | SC<sup>a</sup> | sc-629 |
| Sheep 14–3-3ε | Rabbit IgG (1 µg/ml) | SC | sc-1020 |
| c-Abl | Rabbit IgG (1 µg/ml) | SC | sc-131 |
| Actin | Mouse IgG<sub>2a</sub> (1:500) | Sigma | A4700 |
| Human Akt-1 | Goat IgG (2 µg/ml) | SC | sc-1701 |
| Human Bcl-2 | Mouse IgG<sub>1</sub> (1 µg/ml) | UBI<sup>b</sup> | 05–341 |
| Cbl | Rabbit IgG (1 µg/ml) | SC | sc-1705 |
| Cbl/Cbl-b | Rabbit IgG (1 µg/ml) | SC | sc-1705 |
| Cdc2 | Mouse IgG<sub>2a</sub> (35 ng/ml) | SC | sc-54 |
| CPP32 | Rabbit serum (1:1000) | Pharmingen | 65906E |
| E1B | Mouse IgG (1 µg/ml) | Calbiochem | OP65 |
| Rat ERK1/ERK2 | Rabbit IgG (1 µg/ml) | SC | sc-93 |
| Human focal adhesion kinase | Rabbit IgG (1 µg/ml) | SC | sc-558 |
| Grb2 | Rabbit IgG (1 µg/ml) | SC | sc-422 |
| Human GRK<sup>2</sup> | Rabbit IgG (1 µg/ml) | SC | sc-625 |
| ICH1<sub>a</sub> | Rabbit IgG (1 µg/ml) | SC | sc-625 |
| Human JNK-1 | Rabbit IgG (0.7 µg/ml) | SC | sc-571 |
| Human MEK-1 | Rabbit IgG (1 µg/ml) | SC | sc-219 |
| Human MEK-2 | Rabbit IgG (1 µg/ml) | SC | sc-219 |
| Mouse MEKK1 | Rabbit IgG (1 µg/ml) | SC | sc-252 |
| Mouse MEKK2 | Rabbit IgG (1 µg/ml) | SC | sc-1089 |
| Human MAPK phosphatase-1 | Rabbit IgG (1 µg/ml) | SC | sc-1199 |
| Human c-Myc | Mouse IgG<sub>1</sub> (1 µg/ml) | SC | sc-40 |
| Mouse p53 | Rabbit serum (1:100) | Ref. 16 | |
| Human p53 | Mouse IgG<sub>2a</sub> (2 µg/ml) | Pharmingen | 14471A |
| Human P13K p85 | Mouse IgG<sub>1</sub> (1 µg/ml) | SC | sc-1637 |
| Human P13K p110 | Mouse IgG<sub>1</sub> (1 µg/ml) | SC | sc-602 |
| Phospholipase C-γ | Mouse IgG<sub>1</sub> (0.1 µg/ml) | UBI | 05–3666 |
| Human Raf-1 | Rabbit IgG (1 µg/ml) | SC | sc-132 |
| Human B-Raf | Rabbit IgG (1 µg/ml) | SC | sc-166 |
| Human RasGAP | Rabbit serum (1:3000) | Ref. 35 | |
| Human Src | Rabbit IgG (1 µg/ml) | SC | sc-837 |
| Mouse SEK-1 | Rabbit IgG (1 µg/ml) | SC | sc-18 |
| Mouse TRAP-1<sup>c</sup> | Rabbit IgG (1 µg/ml) | SC | sc-875 |
| Human TX | Goat IgG (2 µg/ml) | SC | sc-1228 |

<sup>a</sup> SC, Santa Cruz Biotechnology.  
<sup>b</sup> UBI, Upstate Biotechnology Inc.  
<sup>c</sup> G protein-coupled receptor kinase 2.  
<sup>d</sup> Mouse tumor necrosis factor receptor-associated protein-1.

Rabbit serum antibodies were detected with protein A conjugated with horseradish peroxidase (1:4000 dilution, Zymed; 10–1023). Goat IgGs were detected with horseradish peroxidase-conjugated rabbit anti-goat antibodies (1:2000 dilution, Zymed; 61–1620). Mouse IgGs were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (1:4000 dilution, Bio-Rad; 170–6516).

Following Fas stimulation. A similar pattern of activation was detected for Raf-1 (Fig. 7A). Among the components of the ERK MAPK pathway, only Raf-1 was cleaved in response to Fas stimulation. Neither B-Raf, MEK1, MEK2, ERK1, nor ERK2 was cleaved in apoptotic cells (Fig. 2). The decline in the activity of Raf-1 and the ERK MAPK pathway correlated with the cleavage of Raf-1 in response to Fas stimulation. To determine whether the reduced activity of the ERK proteins in Fas-stimulated cells resulted from a general down-regulation mechanism, Jurkat cells were stimulated with anti-CD28 antibodies 1 h after Fas cross-linking. However, at later time points, when the Fas-induced ERK response started to decline, the anti-CD28 antibody had no stimulatory effect on the ERK pathway (Fig. 7B). The ERK pathway could still be activated over the Fas-induced response by the anti-CD28 antibody 1 h after Fas cross-linking. However, at later time points, when the Fas-induced ERK response started to decline, the anti-CD28 antibody had no stimulatory effect on the ERK pathway (Fig. 7, compare A and B). There is thus a general impairment of the ERK pathway in Fas-stimulated Jurkat cells. These results suggest that the cleavage of Raf-1 contributes to the shut-off of the ERK MAPK pathway in apoptotic Jurkat cells.

Another potential survival pathway, the PI3K-Akt pathway, appears also to be compromised in apoptotic cells, as assessed by decreased activity of the Akt-1 protein in Fas-stimulated Jurkat cells (Fig. 7A). The decline of the Akt-1 activity follows a similar time course to its degradation after Fas ligation (compare Figs. 2 and 7A). The PI3K subunits were not cleaved in apoptotic cells (Fig. 2). This suggests that the PI3K-Akt pathway is down-regulated as a consequence of Akt1 cleavage.

In contrast, the activities of the p38 and the JNK MAPK pathways remain high even 2 h following Fas ligation (Fig. 7A). Among the components of these pathways that we tested for cleavage in apoptotic cells (MEKK1, MEKK2, SEK-1, JNK-1, p38 MAPK), only MEKK1 was cleaved (Fig. 2). However, this cleavage event does not destroy the kinase activity of the protein but rather leads to an amplification of the apoptotic response (4, 6). Thus, no cleavage event could be determined to negatively affect the activation of the JNK and the p38 MAPK pathways in Fas-stimulated cells. These results demonstrate a selectivity in the turn-off of the ERK and Akt pathways in response to an apoptotic stimulus. Both JNK and p38 have been proposed to have pro-apoptotic functions in different cell types (10), and their sustained activation when ERK and Akt activities have been lost could contribute to the apoptotic signaling in response to Fas ligation.

**DISCUSSION**

The commitment of a cell to mitosis, differentiation, or apoptosis requires the integration of numerous inputs involving multiple signal transduction pathways. For each of these responses, check points during the progression to the final phenotype ensure the proper outcome (10). Thus, the orchestration of signal transduction pathways controls the response of a cell...
to extracellular inputs. Relative to apoptosis, different signaling pathways can contribute to enhancing the death response or inhibit apoptosis and contribute to cell survival (10, 11). A common end point in the commitment to apoptosis is the activation of caspases. Inhibition of caspases generally inhibits apoptosis. However, numerous caspases have been identified, and no one caspase has been shown to be required for apoptosis in all tissues. The prediction is that different caspases differentially contribute to the apoptotic program in different cell types. For example, the targeted deletion of CPP32 (caspase-3) causes loss of apoptosis in the developing brain of mice, but thymic selection appears normal (25), suggesting that CPP32 is necessary for normal brain development but not T cell selection.

Caspase-dependent cleavage can inactivate proteins involved in repair mechanisms or the cell cycle (such as poly-(ADP-ribose) polymerase, DNA-dependent protein kinase, Rb, protein kinase Cα), lead to the degradation of structural proteins (such as lamins and actins), or activate proteins to become proapoptotic (such as p21-activated kinase, MEKK1, gelsolin, and the caspases themselves) (2, 4, 6, 7, 9). Because of the role of various intracellular pathways in the control of the apoptotic response (10), we were interested in determining whether signaling proteins could be cleaved in apoptotic cells. We have found that among 30 proteins with signaling functions, only a limited set is cleaved in apoptotic Jurkat T cell lymphomas and U937 myeloid cells. Our findings show that component members of signaling pathways involved in cell growth and survival can be cleaved in cells undergoing cell death. We demonstrate, for the first time, that RasGAP, Cbl/Cbl-b, Akt-1, and Raf-1 are degraded during apoptosis. The loss of these proteins during apoptosis is consistent with a hypothesis that the caspases turn off survival signals in addition to activating death signals. For example, Akt-1 and the ERK pathway activated by Raf-1 have clearly been shown to promote survival in different cell types (11, 14, 19). Our studies demonstrate that the ERK and Akt activities are inhibited during apoptosis. The role of Cbl/Cbl-b degradation is less clear. Cbl has been shown to function as an adapter protein and to bind PI3K (26). Thus, Cbl can presumably lead to the activation of Akt-1, a kinase mediating survival responses in several cell types (11). The role of RasGAP cleavage is also not immediately obvious. However, Ras, even though it can regulate Raf-1 and lead to ERK activation, has been shown to enhance apoptotic responses (10, 27). Also, targeted disruption of RasGAP has been shown to result in enhanced apoptosis in the brains of developing mouse embryos (28), consistent with the idea that loss of RasGAP contributes positively to apoptosis.

Of the five proteins, RasGAP, Cbl/Cbl-b, Raf-1, and Akt-1, that we newly describe as being degraded during apoptosis, only RasGAP was cleaved in vitro using Jurkat cell lysates that contain high DEVD-directed caspase activity. In the RasGAP primary amino acid sequence, there is the sequence 452DTVD455G that would be efficiently recognized by DEVD-directed caspases. Cleavage at this site would generate a N-terminal fragment that is almost identical to a RasGAP consensus sequence in apoptotic Jurkat T cell lymphomas and U937 myeloid cells. Our findings show that component members of signaling pathways involved in cell growth and survival can be cleaved in cells undergoing cell death. We demonstrate, for the first time, that RasGAP, Cbl/Cbl-b, Akt-1, and Raf-1 are degraded during apoptosis. The loss of these proteins during apoptosis is consistent with a hypothesis that the caspases turn off survival signals in addition to activating death signals. For example, Akt-1 and the ERK pathway activated by Raf-1 have clearly been shown to promote survival in different cell types (11, 14, 19). Our studies demonstrate that the ERK and Akt activities are inhibited during apoptosis. The role of Cbl/Cbl-b degradation is less clear. Cbl has been shown to function as an adapter protein and to bind PI3K (26). Thus, Cbl can presumably lead to the activation of Akt-1, a kinase mediating survival responses in several cell types (11). The role of RasGAP cleavage is also not immediately obvious. However, Ras, even though it can regulate Raf-1 and lead to ERK activation, has been shown to enhance apoptotic responses (10, 27). Also, targeted disruption of RasGAP has been shown to result in enhanced apoptosis in the brains of developing mouse embryos (28), consistent with the idea that loss of RasGAP contributes positively to apoptosis.

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Both MEKK1 and RasGAP are cleaved with kinetics similar to CPP32 (caspase-3) cleavage and activation, indicating that they are primary substrates for caspases. We are currently determining whether the RasGAP aspartic acid at residue 455 is indeed the caspase cleavage site and if RasGAP cleavage contributes to apoptosis.

The fact that Cbl/Cbl-b, Akt-1, and Raf-1 were not cleaved in the in vitro assay using Jurkat lysates indicates that they are not DEVD-directed caspase substrates. They could be substrates either for other caspases or for non-caspase proteases that are not readily assayed in lysates from Fas-stimulated Jurkat cells. We are currently addressing this question biochemically, but in both Jurkat and U937 cells caspase activation is required for Cbl/Cbl-b, Akt-1, and Raf-1 degradation during apoptosis.

In summary, we have identified five additional proteins that are degraded during apoptosis. One of these proteins, RasGAP, is a newly identified DEVD-directed caspase substrate. Cleavage of Raf-1 and Akt-1 inhibits their kinase activity, which may explain why the potential survival signals involving ERK and Akt activity are turned off. Components of the JNK and the p38 MAPK pathways, known to have pro-apoptotic functions in Jurkat cells (31–34), were not cleaved during apoptosis; the exception is MEKK1, whose cleavage does not inhibit its activity. This suggests that caspases function in part to inhibit potential survival signals, allowing the death signals to predominate. The results are consistent with different sets of proteins being cleaved at various stages of the apoptotic re-

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C. Widmann, manuscript in preparation.
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sponse using different proteases in a temporally ordered manner to commit cells to death.

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