Cdc42 Is a Substrate for Caspases and Influences Fas-induced Apoptosis*

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Fas-mediated apoptosis results in the activation of caspases, which subsequently cleave cellular substrates that are essential for normal cell viability. In the present study, we show that the Ras-related GTP-binding protein Cdc42 is susceptible to caspase-catalyzed proteolysis in a number of cell lines, including NIH3T3 fibroblasts, human breast cancer cells (e.g. T47D), and COS-7 cells. Both caspase-3 and caspase-7 were able to catalyze the cleavage of Cdc42, whereas caspase-6 and caspase-8 were without effect. The susceptibility to the caspase-stimulated degradation is specific; although Rac can also serve as a caspase substrate, neither Rho nor Ras is degraded. Caspase sensitivity is conferred by a consensus sequence (DXXD) that lies immediately upstream of the Rho insert regions (residues 122–134) of Cdc42 and Rac. The removal of a stretch of residues (120–139) that includes the insert region or site-directed mutagenesis of either aspartic acid 118 or 121 within a constitutively active background (i.e. Cdc42[F28L]) as well as a wild-type Cdc42 background yields Cdc42 molecules that provide a marked protection against Fas ligand-induced apoptosis. Overall, these results are consistent with a model in which Cdc42 acts downstream of Fas, perhaps to influence the rate of apoptosis, with the ultimate caspase-mediated degradation of Cdc42 then allowing for a maximal apoptotic response.

Cdc42, a Rho-related member of the Ras superfamily, acts as a GTP-binding protein/molecular switch to control a diversity of cellular processes, including the actin cytoskeletal architecture, cell polarity and motility, cell cycle progression, and gene transcription (1). Recently Cdc42 has also been implicated in membrane trafficking (2) with this role being linked to its ability to induce malignant transformation. Slight perturbations in the GTP-binding/GTPase cycle of Cdc42 can give rise to a transformed phenotype (3, 4), and it appears that multiple target/effectors are required. Recently Cdc42 and the related GTP-binding protein Rac have been suggested to play a role in suppressing programmed cell death (5, 6). Therefore, it is an intriguing possibility that at least part of the ability of Cdc42 to transform cells is linked to a circumvention of apoptosis.

A possible link between Cdc42 and the suppression of apoptotic signals may be the serine/threonine kinase p21-activated kinase (PAK).1 It has been well established that PAK is a primary target for the Cdc42 and Rac GTP-binding proteins with its serine/threonine kinase activity being markedly stimulated by activated versions of the GTP-binding proteins (7, 8). It has also recently been shown that PAK1 phosphorylates Bad, a proapoptotic member of the Bcl-2 family (5, 6). In addition, PAK1 is known to stimulate NFkB activity, thus protecting cells against apoptosis (9, 10). Given the increasing indications that PAK activation leads to cell survival and evasion from apoptosis, it becomes attractive to consider a role for its upstream activator, Cdc42, in similar processes.

Thus, in the present study, we examined whether Cdc42 might influence apoptotic signaling pathways, perhaps by performing some type of survival function. We show here that Cdc42 serves as a caspase substrate with the cleavage site lying just upstream of the Rho insert region. Deletion of residues 120–139 or specific point mutations at sites immediately upstream of the insert yields a Cdc42 molecule that significantly retards the ability of apoptotic stimuli (e.g. Fas ligand) to give rise to programmed cell death.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Cell Extracts—COS-7 and NIH3T3 cells were maintained in a humidified 7% CO2 environment in Dulbecco’s modified medium supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Inc.). The media for COS-7 and NIH3T3 cells were supplemented with 10% fetal bovine serum. The human breast cancer cell line T47D was cultured in RPMI 1640 supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Inc.). The media for T47D cells were supplemented with 10% calf serum. COS-7 or T47D cells were washed with ice-cold phosphate-buffered saline and then resuspended in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM MgCl2, 1 mM EDTA, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) for 15 min at room temperature prior to use.

Fas Activation—T47D cells (1–2 × 105) were suspended in 1 ml of RPMI 1640 containing 10% calf serum to minimize incubation volumes. The cells were incubated without serum for 4 h at 37 °C. After the incubation, 50 ng/ml of activating Fas ligand (FasL) (obtained from Upstate Biotechnology Inc.) was added, and cells were incubated for various periods of time. After 1 h, 2 ml of RPMI 1640 without serum were added to cells.

COS-7 or NIH3T3 cells were seeded in 6-well plates in 2 ml of DMEM containing 10% fetal bovine serum. The cells were then incubated without serum for 10 h at 37 °C. After the incubation, 100 ng/ml of activating Fas ligand was added, and cells were incubated for varying periods of time. After 2 h, 1 ml of DMEM without serum was added to adherent cells.

Molecular Constructs—Point mutants were generated using the polymerase chain reaction from a cDNA-encoding Cdc42 that had been subcloned into the BamHI/EcoRI site of pGEM3z. The expression of recombinant proteins in Escherichia coli was performed as described.

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¶ The abbreviations used are: PAK, p21-activated kinase; FasL, Fas ligand; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]1-propanesulfonic acid; HA, hemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase; PBD, p21-binding domain from PAK.

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previously (11). For transient expression in COS-7 cells, the cDNAs encoding the GTP-binding proteins were subcloned into the hemagglutinin (HA)-tagged pcDNA3 vector by using the BamH1/EcoRI restriction sites. For stable expression in NIH3T3 cells, constructs were subcloned into the HA-tagged pLX vector using the same restriction sites.

Assessing Cellular Apoptosis—Stable cell lines were cultured on du-
al-chamber microscope slides (Nunc) for 1 day in normal media and then serum-starved for 8 h. Cells were then treated with 100 ng/ml of FasL as described previously (12). The media were removed, and the cells were placed at 4 °C and gently washed with 10 ml of phosphate-buffered saline. Trypsin (1 ml) was then added to remove adherent cells. The cells were assayed for apoptosis using the Annexin V FITC apoptosis detection kit (Oncogene Research Products) according to the manufacturer’s procedures. Annexin V and propidium iodine (Molecular Probes) staining were used to test for membrane blebbing as described previously (13).

Assays of Caspase-catalyzed Cleavage of Cdc42 and Related Proteins—Typically ~20 μg of lysate proteins from cells expressing HA-tagged Cdc42, Rac1, or RhoA were mixed with 50 ng of caspase-3, caspase-6, caspase-7, or caspase-8 in a total volume of 100 μl. Caspases were purchased from PharMingen. Reactions were carried out in a final concentration of 20 mM HEPES, pH 7.4, 100 mM NaCl, 4 mM EDTA, 20% sucrose, and 0.1% CHAPS. The reactions were performed at room temperature for varying times in the presence or absence of the tetrapeptide inhibitor Ac-DEVD-CHO (200 ng). Aliquots (25 μl) were removed from the reaction mixture, and the reaction was stopped by boiling for 5 min in 6 × SDS-polyacrylamide gel electrophoresis sample buffer. Cdc42 and other GTP-binding proteins were detected by Western blot analysis using a 1:1000 dilution of anti-HA (HA 1.11) monoclonal antibody purchased from Covance Research Products.

Measurement of Caspase Activities in Cell Lysates—Cells (1 × 10⁶) were plated onto 6-well plates and starved for 12 h in 1% DMEM. The cells were then lysed in 50 mM Tris, pH 7.2, 1 mM EDTA, 10 mM EGTA, and 1% Nonidet P-40. Fifty μg of lysate proteins were incubated with 10 μM Ac-DEVD-(7-amino-4-trifluoromethylcoumarin) (purchased from PharMingen) in 1 ml of 20 mM PIPES, pH 7.2, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, and 10% sucrose. Fluorescence was then monitored using a PerkinElmer Life Sciences LS-5 fluorescence spectrophotometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase-8 activity was measured in an identical fashion but using 10 μM Ac-IETD-(7-amino-4-trifluoromethylcoumarin) (purchased from PharMingen).

Assaying the Activation of Cdc42 in Cells Using the Limit Cdc42/Rac-binding Domain from PAK—The activation of cellular Cdc42 was assayed as described previously (14) based on a procedure originally developed for Ras (15). COS-7 cells were transiently transfected with the cDNA for wild-type Cdc42 in the pcDNA3 vector. Cells were allowed to grow in the presence of 10% fetal bovine serum for 24 h and then starved for 4 h followed by stimulation with 100 ng/ml epidermal growth factor or 100 ng/ml FasL. Typically ~20 min after stimulation, cells were lysed in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 20 mM β-glycerol phosphate, 20 μM GTP, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotonin and then incubated with 50 μg of GST-PBD (for p21-binding domain from PAK). The cells were rocked at 4 °C for 3 h. GST-PBD was then precipitated with glutathione-agarose beads, washed three times with lysis buffer, and subjected to SDS-polyacrylamide gel electrophoresis and immuno-

RESULTS

Effects of Cdc42 on FasL-induced Apoptosis—It has recently been shown that the proapoptotic Bad protein can be phospho-

rylated by PAK1 (5, 6), thereby preventing its interaction with Bel-2 or Bcl-xL. Given that Cdc42 is a potent activator of PAK (7, 8, 16), we set out to examine whether wild-type Cdc42 or

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different Cdc42 mutants might be capable of influencing apoptotic responses. Several different constructs of Cdc42 were stably transfected into NIH3T3 cells that were subsequently treated with the proapoptotic ligand FasL. We found that a Cdc42 construct (designated \(\text{DL8}^{\text{D}}\)) in which loop 8 from H-Ras (residues 121–127) was substituted for amino acids 120–139 in Cdc42, including a stretch of amino acids unique to Rho-related proteins but missing in Ras (the Rho insert, residues 122–134), protected cells against FasL-induced apoptosis (Fig. 1). Even under conditions in which a constitutively active form of Cdc42 (Cdc42\((\text{F28L})\)) that is potently transforming (3) did not show significant protective effects, the \(\text{DL8}^{\text{D}}\) deletion in a constitutively active background (designated Cdc42\((\text{F28L})\)\(\text{DL8}^{\text{D}}\)), which yielded an activated but transformation-defective Cdc42 molecule (17), showed strong protection. Moreover, the \(\text{DL8}^{\text{D}}\) deletion in a wild-type background also showed substantial protection against apoptosis. The quantitation of apoptosis using Annexin V FITC/propidium iodine staining showed that under conditions in which at least 80% of the (vector) control cells underwent apoptosis following treatment with FasL, typically only 20–30% of the cells died when expressing the Cdc42\(\text{DL8}^{\text{D}}\) constructs. These results suggested that the \(\text{DL8}^{\text{D}}\) deletion somehow provided Cdc42 with a significant protective capability.

**Cdc42 Is Susceptible to Degradation in Response to Apoptotic Stimuli**—We have found that Cdc42 is subject to degradation when cells are exposed to a variety of types of apoptotic treatments. For example, upon the transient expression of HA-tagged Cdc42 in NIH3T3 fibroblasts, COS-7 cells, and human breast cancer T47D cells followed by treatment with FasL, the HA-tagged Cdc42 underwent a time-dependent degradation. Fig. 2A, left panel, shows the results obtained upon treating cells with FasL and then monitoring Cdc42 by Western blotting with an anti-HA antibody, whereas the right panel shows the corresponding controls (no FasL treatment). There was significant variability in the time period for degradation, depending on the cell type, with COS-7 cells requiring a much greater time period for achieving complete degradation as compared with T47D or NIH3T3 cells. This most likely reflects differences in the relative amount of caspase activity in the individual cell types.

**Cdc42 Is Sensitive to Caspases**—We have found that Cdc42 is subject to degradation when cells are exposed to a variety of types of apoptotic treatments. For example, upon the transient expression of HA-tagged Cdc42 in NIH3T3 fibroblasts, COS-7 cells, and human breast cancer T47D cells followed by treatment with FasL, the HA-tagged Cdc42 underwent a time-dependent degradation. These results suggested that the \(\Delta L8\) deletion somehow provided Cdc42 with a significant protective capability. Among the possible explanations for these findings was that Cdc42 might itself be a target for apoptotic factors (e.g., caspases) such that the \(\Delta L8\) deletion reduced the susceptibility of Cdc42 and thereby slowed the overall time course for programmed cell death.
Cdc42 is a Substrate for Caspases—Next we examined which caspases cleave Cdc42 or with the different indicated Cdc42 mutants. The cells were serum-starved for 10 h and then treated with FasL (100 ng/ml) for the indicated periods of time. The degradation of Cdc42 was then analyzed by Western blotting with anti-HA antibody.

The case in T47D cells (data not shown). Rac is also susceptible to the degradation induced by apoptotic stimuli, although typically it appears to be a less effective substrate than Cdc42 (e.g. see Fig. 2B). On the other hand, we did not find either Ras or RhoA to be degraded in any of the cell types examined following treatment with apoptotic stimuli. An example is shown for the treatment of NIH3T3 cells with FasL (Fig. 2C), and similar results were obtained with COS-7 and T47D cells (data not shown). Thus, there appears to be a rather striking selectivity in terms of the small GTP-binding proteins that are sensitive to degradation.

Cdc42 Is a Substrate for Caspases—Next we examined which specific caspases cleave Cdc42 and Rac. Lysates from COS-7 cells expressing HA-tagged Cdc42 or Rac were treated with a number of different purified recombinant caspases (e.g. caspase-3, caspase-6, caspase-7, and caspase-8). We found that both Cdc42 and Rac were effectively degraded by caspase-3 and caspase-7, whereas neither GTP-binding protein was susceptible to caspase-6 or caspase-8 (Fig. 3A). The addition of a caspase inhibitor peptide, DEVD, to the lysates blocked the degradation of both Cdc42 and Rac (see Fig. 3B; in these experiments, tissue transglutaminase was examined as a negative control). Overall, these findings are consistent with the notion that caspase-3 and caspase-7 are effector caspases (i.e. downstream participants in caspase cascades), whereas caspase-6 and caspase-8 typically serve as initiator caspases (18). We also examined whether the cleavage of Cdc42 was nucleotide-dependent and thus transiently expressed wild-type (i.e. predominantly GDP-bound) Cdc42 in COS-7 cells as well as a nucleotide-depleted Cdc42 mutant (Cdc42(T17N)) and a GTPase-defective, dominant-active Cdc42 mutant (Cdc42(Q61L)). We found that each of these Cdc42 proteins was cleaved at essentially the same rate (Fig. 4). Thus, the caspase susceptibility of Cdc42 must involve a site (or sites) that is distinct from those influenced by the GTP-dependent conformational changes that underlie the molecular switch function of GTP-binding proteins.

We have also performed experiments examining the ability of recombinant caspases to directly catalyze the degradation of recombinant Cdc42. GST-Cdc42, -Rac, and -RhoA were incubated with different purified recombinant caspases and then analyzed by Western blot analysis with anti-GST. We found that caspase-3 was able to effectively degrade Cdc42 as read out either by using an anti-GST antibody (Fig. 5) or by using an anti-Cdc42 carboxyl-terminal antibody (data not shown). Under these conditions, GST-Rac was weakly degraded by caspase-3 (relative to the degradation observed for Cdc42), and GST-RhoA showed no detectable degradation. Other purified recombinant caspases, such as caspase-6 or caspase-8, were ineffective against all of the GTP-binding proteins tested. The necessity of adding caspase-7 to cellular lysates to catalyze Cdc42 degradation, coupled with the finding that purified caspase-3 effectively degrades Cdc42, suggests that caspase-7 may be acting upstream of caspase-3 in a pathway leading to Cdc42 proteolysis. Other caspases, aside from caspase-3, may also be acting downstream from caspase-7 given that both caspase-7 and caspase-3 are able to strongly catalyze the degradation of Rac in cellular lysates, whereas the direct addition of purified caspase-3 to Rac shows a significantly slower degradation.

Identification of the Cleavage Sites on Cdc42—Sequence analysis of Cdc42 and Rac revealed a caspase consensus sequence (DXXD motif) located between residues 118 and 121 just upstream of the Rho insert region (Fig. 6). Neither Rho nor Rac appears to contain a caspase consensus sequence, which is consistent with our findings that these GTP-binding proteins do not serve as substrates for proteolysis. We then used site-directed mutagenesis to alter the putative caspase cleavage sites on Cdc42 with the following point mutants being gener-
ated: Cdc42(D118N), Cdc42(D121N), and Cdc42(D122N). The HA-tagged wild-type Cdc42 and the HA-tagged Cdc42 mutant proteins were stably expressed in NIH3T3 cells and then analyzed for FasL-induced degradation. Both wild-type Cdc42 and the Cdc42(D122N) mutant were effectively degraded under conditions of FasL-induced apoptosis (Fig. 7), whereas the Cdc42(D118N) and Cdc42(D121N) mutants were resistant to degradation.

Similar results were obtained when purified caspases were added to lysates from NIH3T3 cells that stably expressed the different HA-tagged Cdc42 proteins. We found that wild-type Cdc42 and the Cdc42(D122N) mutant were susceptible to degradation stimulated by either caspase-7 or caspase-3 (Fig. 8), whereas the Cdc42(D118N) and Cdc42(D121N) mutants were resistant to degradation under each condition. The same was true when examining the ability of recombinant caspase-3 to catalyze the degradation of the different purified recombinant GST-Cdc42 proteins (data not shown). Thus, we conclude that aspartic acid residues 118 and 121 within Cdc42 are essential sites for caspase-catalyzed proteolysis.

Caspase-insensitive Cdc42 Mutants Block Fas-mediated Apoptosis—Having identified the essential sites on Cdc42 for degradation by caspases, we next investigated whether the Cdc42 mutants that were resistant to caspase-mediated proteolysis would be able to block Fas-mediated apoptosis.

**Fig. 9.** Cdc42 point mutants that are caspase-insensitive inhibit apoptosis. A, NIH3T3 fibroblasts stably expressing Cdc42(F28L), Cdc42(F28L/D118N), Cdc42(F28L/D121N), or Cdc42(F28L/D122N) were serum-starved in DMEM with 1% calf serum and then treated with 100 ng/ml FasL for 4 h. Apoptosis was assayed by staining with Annexin V/propidium iodine. The results represent the analysis of 500 cells (n = 6). B, an identical set of experiments as shown in panel A except that the NIH3T3 cells were stably expressing Cdc42(D118N), Cdc42(D121N), and Cdc42(D122N). In all cases, the control represents cells transfected with vector alone. C, human breast cancer cells T47D transiently expressing Cdc42(D118N), Cdc42(D121N), or Cdc42(D122N) were serum-starved in DMEM with 1% calf serum and then treated with 50 ng/ml FasL. After 12 h, the cells were analyzed for apoptosis by staining with Annexin V/propidium iodine. *wt*, wild type.
caspase-mediated degradation, we then examined whether these caspase-insensitive mutants could influence the ability of FasL to induce apoptosis. We first established NIH3T3 cell lines that stably expressed the activated Cdc42(F28L) mutant and different Cdc42 molecules that were point-mutated within an F28L background (i.e. Cdc42(F28L/D118N), Cdc42(F28L/D121N), and Cdc42(F28L/D122N)) as we have done in the past to assess the role of Cdc42(F28L) in cell growth and actin cytoskeletal rearrangements (2–4, 17). Fig. 9A shows the results obtained when the different stable cell lines were treated with 100 ng/ml FasL and then scored for apoptosis using dye-binding assays (as described under “Experimental Procedures”). Consistent with other results (e.g. Fig. 1), cells expressing the constitutively active Cdc42(F28L) mutant showed essentially the same susceptibility to FasL-induced apoptosis as control cells expressing vector alone. Cells expressing the Cdc42(F28L/D122N) double mutant also showed little protection against FasL-mediated cell death. However, cells expressing either of the caspase-insensitive mutants, Cdc42(F28L/D118N) or Cdc42(F28L/D121N), showed significant resistance against programmed cell death. It is interesting that similar results were obtained when the point mutations were generated within a wild-type Cdc42 background. The stable expression of either Cdc42(D118N) or Cdc42(D121N) conferred resistance to FasL-induced cell death in NIH3T3 cells, whereas the Cdc42(D122N) mutant was essentially ineffective (Fig. 9B). This was also the case when human breast cancer cells (T47D) were transiently transfected with the corresponding cDNAs for these different Cdc42 mutants (Fig. 9C).

Fig. 10A shows the dose-response profiles for FasL-induced apoptosis in NIH3T3 cells that stably express the various point mutants in the Cdc42(F28L) background, whereas Fig. 10B shows the corresponding profiles for fibroblasts expressing the different point mutants in the wild-type Cdc42 background. We see that in both cases, a substantial protection is provided by Cdc42 molecules containing either the D118N or the D122N mutation at FasL levels ≤ 100 ng/ml. However, at higher levels of FasL (200 ng/ml), the protection effects are greatly diminished. Similarly, in time course experiments, the D118N and D122N mutants show substantial protection up to 24 h of treatment with 100 ng/ml FasL, whereas at longer times (>36 h), the protective effects by the Cdc42 mutants were signifi-

Fig. 10. Dose-response profiles for FasL-induced apoptosis. A, NIH3T3 fibroblasts that stably express Cdc42(F28L/D118N), Cdc42(F28L/D121N), Cdc42(F28L/D122N), or empty vector (controls) were serum-starved in DMEM with 1% calf serum and then treated with the indicated doses of FasL for 24 h. Apoptosis was assayed by staining with Annexin V/propidium iodine. The results represent the analysis of 500 cells (n = 6). B, an identical set of experiments as shown in panel A except using cells that express Cdc42(D118), Cdc42(D121N), and Cdc42(D122N). Conc, concentration.

Fig. 11. Relative expression of Fas and other apoptotic signaling components. NIH3T3 fibroblasts stably expressing wild-type HA-tagged Cdc42(D118N), Cdc42(D121N), Cdc42(D122N), or empty vector were subjected to Western blot analysis to assess the relative levels of expression of Fas, Fas-associated death domain (FADD), caspase-7, and caspase-3.
cantly diminished (data not shown). These data indicate that the protective effects provided by Cdc42 are not irreversible and can be overcome by increasing the strength and the time duration of the apoptotic stimuli.

It has been reported that Ras has the ability to inhibit FasL-mediated apoptosis through the down-regulation of Fas expression (21). However, as shown in Fig. 11, there is no detectable effect on Fas expression in NIH3T3 cells that express those Cdc42 mutants that give rise to protection against FasL-mediated apoptosis. Likewise, no detectable effects on the expression of the Fas-signaling participants Fas-associated death domain, caspase-3, or caspase-7 were found.

We also examined whether the expression of the Cdc42 mutants that exhibited protection against FasL-induced apoptosis had any effect on the cellular activities of caspases. Fig. 12, A and B, shows that FasL stimulated the activation of caspase-3 and caspase-8 in NIH3T3 cells with maximal activation occurring in 3–4 h followed by a steady decline in activity, consistent with previous reports (22). The FasL-stimulated caspase-3 activity in cells expressing each of the Cdc42 mutants tended to be higher (sometimes by as much as 50–60%) rather than lower than the activity measured in control NIH3T3 cells (Fig. 12A). When assaying caspase-8, which is upstream of caspase-3, we did observe minor reductions in the activity compared with control cells (Fig. 12B). However, in some experiments we found no detectable differences under conditions in which significant protection against apoptosis was provided by the D118N and D121N mutants, thus indicating that the protective effects cannot be attributed to a change in the expression or function of caspase-8.

The fact that caspase-insensitive mutations in a wild-type Cdc42 background were still able to confer protection against FasL-induced apoptosis was somewhat surprising. Thus, we hypothesized that treatment with FasL is able to promote Cdc42 activation. In fact, various Rho family members including Cdc42 have been reported to be activated by FasL (19, 20). We have examined this under conditions in which Cdc42 is able to protect against apoptosis and found that FasL was able to induce a weak but consistent stimulation of Cdc42 activation as read out by the interaction of GTP-bound Cdc42 with the limit-binding domain of one of its primary targets PAK (Fig. 13). However, the overall extent of activation of Cdc42 by FasL was significantly reduced compared with other activating ligands (e.g. epidermal growth factor (Fig. 13)). Thus, it appears that FasL may only activate a small percentage of the total cellular
pool of Cdc42 but that this may be sufficient to have an impact on FasL-induced apoptosis.

**DISCUSSION**

It is becoming increasingly clear that the signaling pathways responsible for programmed cell death are every bit as complex as those signaling activities that stimulate cell cycle progression and mitogenesis. Moreover, there is every reason to believe that apoptotic ligands/factors will initiate a diversity of signaling outputs, some of which will have the expected stimulatory effects on apoptosis, whereas others will exert opposing effects, perhaps providing some modulation to the overall rate at which cell death proceeds. This is similar to what has emerged from studies of the signaling pathways responsible for mitogenesis. For example, the growth factor-dependent activation of Ras stimulates cell cycle progression but also leads to the expression of genes that negatively regulate cell growth and even contribute to apoptosis (reviewed in Ref. 23). Thus, for these reasons, we felt that it was of interest to examine how Cdc42, a Rho-related member of the Ras superfamily that has been implicated in a number of fundamental cellular processes including cell cycle progression, cell shape, and motility, influences programmed cell death.

We show here that the deletion of a stretch of residues that included the Rho insert region yielded a Cdc42 molecule that was able to significantly retard the rate of apoptotic progression. We have gone on to show that immediately upstream from the insert region, Cdc42 contains an essential site for caspase-catalyzed proteolysis. The degradation of Cdc42 in cells occurs under conditions of apoptotic stimulation and appears to be specific. Rac, which is highly related to Cdc42, can also serve as a caspase substrate, but neither Rho nor Ras was degraded during Fas-mediated apoptosis in any of the cell lines examined. Mutating the essential sites for caspase-catalyzed proteolysis yields Cdc42 molecules that can markedly reduce the susceptibility of cells to apoptosis, most likely by slowing their rate of progression toward cell death. What is especially interesting is that Cdc42 molecules, which are not constitutively active but nonetheless are mutated at the caspase-sensitive sites, exhibit strong antiapoptotic effects.

This effect then points to apoptotic ligands such as FasL as potentially having the capability for promoting the activation of Cdc42. Various reports have appeared suggesting that FasL and related ligands (e.g. tumor necrosis factor-α) are able to stimulate the activation of Cdc42 (18, 24). We have carefully examined this under conditions in which Cdc42 influences apoptotic responses and find that FasL is able to increase the levels of activated Cdc42 in cells, but this appears to be a rather modest effect, apparently representing a small percentage of the total cellular pool of Cdc42. Therefore, in order for Cdc42 to influence Fas-mediated apoptosis, some type of signal amplification may be required.

Fig. 14 depicts a working model that is consistent with the results presented here. Fas activation is shown to initiate at least two types of pathways, one that leads to the activation of caspases and a classical apoptotic response and a second that serves to oppose the apoptotic signal (i.e. similar to a survival activity). This is similar to what has been proposed for the related apoptotic ligand tumor necrosis factor-α (25–27). The activation of Cdc42 and/or one of its downstream effectors would be essential for the survival pathway. It appears that the effects of Cdc42 are not directed at the level of caspase expression or activation. Rather, both caspase-3 and its upstream activator caspase-8 were strongly stimulated by FasL under conditions in which caspase-insensitive Cdc42 mutants were able to provide strong antiapoptotic effects. Thus, the protective/survival effects mediated by Cdc42 must be directed at events occurring downstream from caspase activation, perhaps influencing the susceptibility of key substrates, the proteolysis of which is necessary for a full commitment to apoptosis. The ability of FasL to both initiate caspase activation and promote Cdc42-mediated survival signals might then provide for a certain time frame for the apoptotic response to occur. As caspase activation becomes maximal and the Cdc42 degradation ensues, the apoptotic signaling pathway(s) would in effect take over and allow the completion of the cell death program.

The expression of the constitutively active Cdc42(F28L) mutant did not cause a significant slowing of the Fas-induced apoptotic response. However, clearly an advantage is gained by expressing Cdc42 molecules that are not susceptible to caspase-catalyzed degradation. At the present time, we have not identified the downstream Cdc42 targets that are responsible for providing a survival signal. However, two attractive candidates are PAK and NFκB, both of which have been demonstrated to participate in Cdc42-mediated signaling events and to contribute to antiapoptotic responses (5, 6, 9). Future work will be directed toward delineating the Cdc42 signaling pathways operating in Fas-induced responses and to establish whether any connection exists between Cdc42-mediated survival responses and the ability of Cdc42 to cause the malignant transformation of cells.

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