Molecular Biology of the Cell
Vol. 8, 1687–1698, September 1997

The Small GTPase Cdc42 Initiates an Apoptotic Signaling Pathway in Jurkat T Lymphocytes

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Submitted March 24, 1997; Accepted June 18, 1997
Monitoring Editor: Joan Brugge

Apoptosis plays an important role in regulating development and homeostasis of the immune system, yet the elements of the signaling pathways that control cell death have not been well defined. When expressed in Jurkat T cells, an activated form of the small GTPase Cdc42 induces cell death exhibiting the characteristics of apoptosis. The death response induced by Cdc42 is mediated by activation of a protein kinase cascade leading to stimulation of c-Jun amino terminal kinase (JNK). Apoptosis initiated by Cdc42 is inhibited by dominant negative components of the JNK cascade and by reagents that block activity of the ICE protease (caspase) family, suggesting that stimulation of the JNK kinase cascade can lead to caspase activation. The sequence of morphological events observed typically in apoptotic cells is modified in the presence of activated Cdc42, suggesting that this GTPase may account for some aspects of cytoskeletal regulation during the apoptotic program. These data suggest a means through which the biochemical and morphological events occurring during apoptosis may be coordinately regulated.

INTRODUCTION

Physiologically regulated, or programmed cell death, generally occurs through a series of defined morphological and nuclear changes known as apoptosis. Although initiated by diverse stimuli, apoptotic cells are characterized by contraction of the cell body, extensive membrane blebbing, nuclear condensation, and DNA fragmentation (reviewed in Ellis *et al.*, 1991; Martin *et al.*, 1994; Kerr, 1995)). This process of controlled cell death enables the organism to effectively remove the dead cells by noninflammatory mechanisms, and is known to be an important mechanism for maintenance of homeostasis and function in diverse biological systems. In the immune system, apoptosis is important in both normal development and in the establishment of immune self-tolerance (Linette *et al.*, 1994; Abbas, 1996). Examples include the removal of unwanted cells after a primary immune response and/or an inflammatory response, and the elimination of self-reactive T cells; both processes are mediated via an apoptotic process (Linette and Korsmeyer, 1994; Nagata and Golstein, 1995; Abbas, 1996; Rathmell *et al.*, 1996). Apoptosis is also used by cytotoxic T lymphocytes to eliminate target cells (Kägi *et al.*, 1994).

Certain cell surface receptors involved in maintenance of the immune system seem to be directly coupled to the cell death machinery. Fas/Apo-1 (CD95) is a member of the tumor necrosis factor (TNF) superfamily. Ligation of Fas by anti-Fas antibodies or its specific ligand induces rapid apoptosis, both in vitro and in vivo (Takahashi *et al.*, 1994; Nagata and Golstein, 1995; Rieux-Laucat *et al.*, 1995; Rathmell *et al.*, 1996). Fas and related receptors of the TNF family directly couple to a recently elucidated ICE protease (or caspase (Alnemri *et al.*, 1996)) cascade that initiates the cell death sequence by as yet unknown mechanisms (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Genetic and biochemical studies have indicated a central role for the aspartate-directed proteases of the caspase family in apoptosis (Ellis *et*

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al., 1986; Martin and Green, 1995; Yuan, 1995; Chinnaiyan and Dixit, 1996; Takahashi and Earnshaw, 1996; Whyte, 1996).

In contrast to those receptors directly linked to the cell death machinery, a variety of other stimuli and/or conditions can cause cells to undergo an apoptotic response. These include the withdrawal of growth factors, with the concomitant loss of growth factor receptor signals, and the presence of "stress" signals. The latter include cytokines, UV radiation, oxidants, heat shock, ischemia, and inflammation (Cosulich and Clarke, 1996; Hannun, 1996; Zanke *et al.*, 1996). Cell death induced by stress signals is usually a much slower process than that induced by Fas and other death receptors, occurring over periods of 12–48 h rather than within 1–4 h. A common feature of many of these apoptotic signals is the generation of intracellular ceramides via the action of membrane-associated sphingomyelinases, and ceramides have been implicated as a cell death signal (Hannun, 1996). Ceramides have also been linked to activation of the stress-activated protein kinases or c-Jun amino terminal kinases (JNKs) (Westwick *et al.*, 1995; Verheji *et al.*, 1996). Increased JNK signaling seems to be an important determinant of apoptotic responses in certain types of cells (Ham *et al.*, 1995; Xia *et al.*, 1995; Chen *et al.*, 1996b; Cosulich and Clarke 1996; Zanke *et al.*, 1996).

The protein kinase cascade that controls JNK activity is regulated by the action of the small GTPases Rac and Cdc42 in a variety of cells (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Zhang *et al.*, 1995). This finding implies that under appropriate circumstances Rac and/or Cdc42 may be important mediators of cell signals leading to apoptotic responses. Our laboratory has recently identified D4 GDI (GDP dissociation inhibitor) as a novel cytoplasmic substrate for CPP32/caspase-3 in Jurkat T cells (Na *et al.*, 1996). D4 GDI is an abundant regulator of Rho family GTPases in lymphocytes and myeloid cells (Lelias *et al.*, 1993; Scherle *et al.*, 1993), serving to modulate the interaction of these GTPases with stimulatory and inhibitory regulatory proteins and effector targets, as well as to determine the intracellular localization of the GTPases (Bokoch and Der, 1993). The cleavage of D4 GDI by ICE proteases during apoptosis may therefore induce altered activity and signaling by Rho family GTPases. These observations prompted us to examine possible effects of Rho GTPases on apoptotic events. In the present report, we describe the ability of Cdc42 to induce apoptosis in Jurkat T lymphocytes by activating the JNK signaling cascade. Morphological alterations resulting from constitutive Cdc42 activation are also observed.

MATERIALS AND METHODS

Plasmids

The tetrapeptide caspase inhibitors, Ac-YVAD-CMK and Ac-DEVD-CHO, were purchased from Bachem (Torrance, CA). Anti-CD95/Fas (CH-11) monoclonal antibody was purchased from Immunotech (Eugene, OR). Ceramide analogues were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Mammalian expression vectors for GTPases, including RhoAQ63L/pCMV5, Rac1Q61L/pCMV5, Cdc42/pCMV5, Cdc42T17N/pCMV5, Cdc42Q61L/pCMV5, and for kinases JNK1/pcDNA3, p38/pcDNA3 were constructed as described in Zhang *et al.* (1995). CrmA/pcDNA3 was obtained from Vishva Dixit (University of Michigan; Tewari and Dixit, 1995). hBcl2/pSSFV was obtained from Stanley Korsmeyer (Washington University, St. Louis, MO; Hockenberry *et al.*, 1993). hCD14/pRc/RSV, an expression vector containing the monocyte-specific cell surface marker CD14, was as described (Lee *et al.*, 1992). Vectors for dominant active and inactive kinases ΔMEKK/pSRα, ΔMEKK(K432 M)/pSRα, and JNKK(K116R)/pSRα were obtained from M. Karin (UCSD, San Diego, CA; Minden *et al.*, 1994, 1995). Constitutively active Raf-1(BxB)/pSRα was a gift from J. Jackson (The Scripps Research Institute). Vectors for the transactivation assays were 5xGal4-luciferase reporter plasmid Gal4-c-Jun(1–223) and Gal4-Elk(83–428) [obtained from M. Karin, UCSD; Minden *et al.*, 1994, 1995)]. All plasmids were prepared from *Escherichia coli* using the Qiagen Plasmid Maxiprep kit (Qiagen, Chatsworth, CA).

Cell Culture, Electroporation, and Selection of Transfected Cells

Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Gaithersburg, MD), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 50 μM β-mercaptoethanol and maintained at 37°C in a 5% CO_2 atmosphere. For transient protein expression, 2×10^7 Jurkat T cells were resuspended in 400 μl RPMI 1640 with 20 ng/μl hCD14/pRc/RSV in the presence or absence of 160 ng/μl GTPase expression vectors or different combinations of expression vectors (as indicated in individual experiments) in a 0.4-cm gap electroporation cuvette (Bio-Rad, Richmond, CA), kept on ice for 15 min, and then electroporated with a Bio-Rad Gene Pulser at 960 μF, 0.3 kV. The electroporated cells were kept on ice for 15 min, resuspended in 12 ml of culture medium, and cultured overnight. To select the transfected cells, cells were pelleted and incubated with anti-CD14 antibody conjugated to metal beads (Miltenyi Biotech, Auburn, CA) in 100 μl RPMI 1640 at 4°C for 25 min. The cells were then passed through a magnetized column (Miltenyi Biotech) to bind the CD14-expressing cells (i.e., the cells that also would be expressing the cotransfected cDNAs). After extensive washing with RPMI 1640 to remove unbound CD14-deficient cells, the column was removed from the magnet and eluted with culture medium to collect the $CD14^+$ cells. The selected cells were diluted to a density of 2×10^5 cells/ml, incubated with stimuli/inhibitors, and analyzed for apoptosis at different time points.

Analysis of CD14 Expression

The efficiency of the electroporation transfections and column selections for $CD14^+$ cells was monitored by flow cytometric analysis. Cells were pelleted and incubated with fluorescein isothiocyanate-conjugated anti-CD14 antibody (PharMingen, San Diego, CA) in a phosphate-buffered saline (PBS) plus 1.5 μg/ml propidium iodide (PI) solution at 4°C for 45 min. The cells were pelleted and resuspended in PBS and analyzed with FACSort (Becton and Dickinson, San Jose, CA). The population of living cells that excluded PI were gated for analyzing the percentage of CD14-expressing cells. We

routinely enriched the cell population to greater than 80% $CD14^+$ cells using the procedures described.

Analysis of Apoptosis

Flow Cytometric Analysis. Apoptosis of cells was assessed by measurement of the fragmentation of chromosomal DNA using flow cytometry as described (Nicoletti *et al.*, 1991). Briefly, cells were pelleted and resuspended in a solution of 20 μg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. In each single analysis, 1×10^4 nuclei were collected and analyzed with FACSort. Nuclei containing a lower amount of DNA than the G_0-G_1 nuclei were considered to be apoptotic cells.

Morphological Analysis. The morphology changes characteristic of apoptotic Jurkat cells were routinely assessed by staining cells in a solution of 2 μg/ml acridine orange plus 2 μg/ml ethidium bromide and visualization with fluorescence microscopy as described (McGahon *et al.*, 1995; Waters *et al.*, 1996). Cytotoxic T cell (CTL) killing, differential interface contrast (DIC) microscopy, and image processing procedures were performed as described previously (Hahn *et al.*, 1994), except that the cells were maintained in a sealed Dvorak chamber heated to 37°C by a temperature-controlled air curtain.

Transactivation and JNK Assays

Jurkat T cells were transfected with a mixture of 40 ng/μl 5xGal4-luciferase reporter plasmid, 60 ng/μl activator plasmids encoding Gal4-c-Jun(1–223), or Gal4-Elk(83–428), and 140 ng/μl of plasmids encoding GTPases or kinases using the procedures described above. The cells were harvested the following day, lysed, and assayed for luciferase activity using the Promega Luciferase assay kit. Readings were normalized to equivalent amounts of cytosolic protein for all experiments. Concentration of cytosolic proteins was determined using the Coomassie Plus Protein Assay (Pierce, Rockford, IL).

JNK activity was determined using glutathione *S*-transferase (GST)-conjugated-c-Jun as substrate for a solid phase assay as described in Zhang *et al.* (1995). Jurkat cells were transfected with the indicated plasmids, and, at the indicated times after selection, 8×10^5 cells were lysed in 100 μl of cell lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonic fluoride, 5 μg/ml aprotinin, 0.5 μg/ml leupeptin). Six micrograms of GST-c-Jun (aa 1–79) coupled to glutathione-Sepharose beads were added to clarified lysates and incubated for 3 h at 4°C. Bead pellets were washed and kinase assays performed as described (Zhang *et al.*, 1995). Quantitation was by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Cdc42 Induces Apoptosis in Jurkat T Cells

In previous studies, we observed that D4 GDI, an abundant regulator of the activity of the Rho GTPase family in Jurkat T cells, is proteolytically degraded when apoptosis is induced in these cells by both receptor and nonreceptor stimuli (Na *et al.*, 1996). Because GTPases of the Rho family are potentially activated as a result of the cleavage of D4 GDI under these conditions, we evaluated whether these GTPases contributed to apoptotic signaling. We prepared constitutively active mutants of RhoA, Rac1, and Cdc42Hs and transiently expressed these in Jurkat T cells. During selection of the cell population expressing the mutant constructs, we observed that the cells transfected with Cdc42(Q61L) were consistently reduced in number when compared with cells transfected with other GTPases or

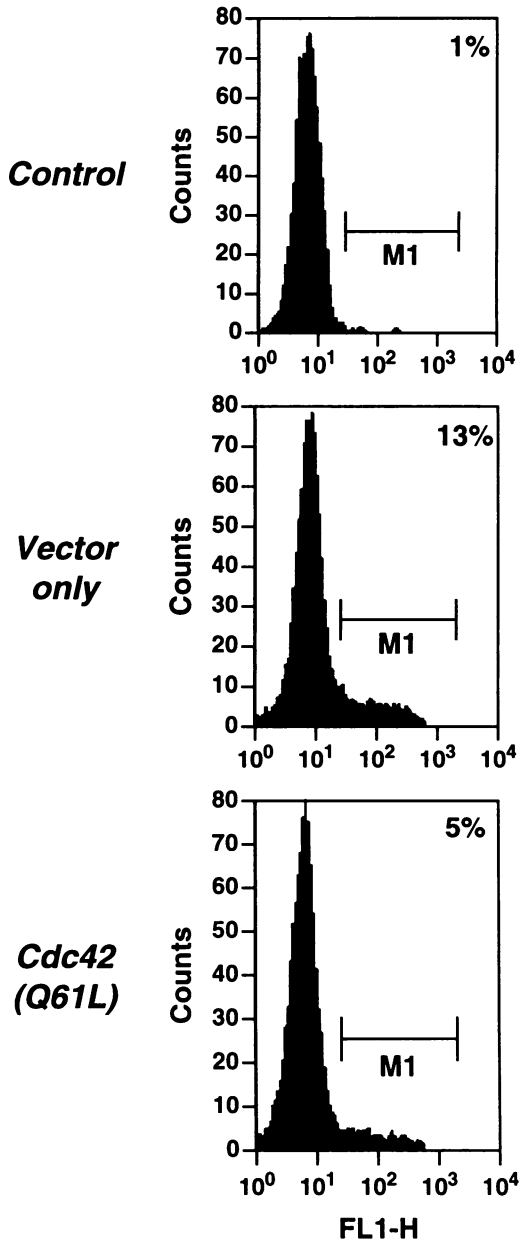

Figure 1. Cytotoxic effect of overexpression of Cdc42(Q61L) in Jurkat T lymphocytes. Jurkat T cells were transfected with the CD14 expression vector hCD14/pRc/RSV in the presence of control pCMV5 vector (middle panel) or plus pCMV5/Cdc42(Q61L) (lower panel), as described in MATERIALS AND METHODS. Control (upper panel) = no cDNA added. Transfection efficiencies were determined by flow cytometric analysis of the percentage of CD14-expressing cells gated as (MI) and are indicated in each panel. Reduced numbers of Cdc42(Q61L)-expressing cells were observed consistently in all experiments.

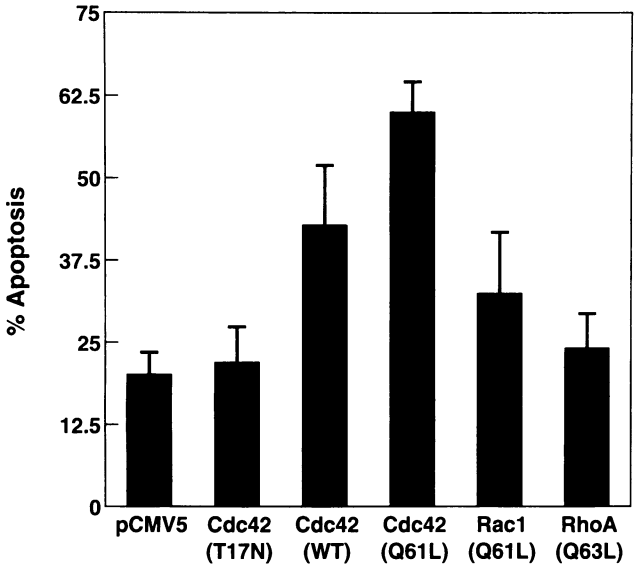

Figure 2. Induction of apoptosis by Rho family GTPases in Jurkat T cells. Jurkat T cells were cotransfected with CD14 and an eightfold cDNA excess of Rho GTPase vectors to ensure expression in all CD14-positive cells. The population was enriched for CD14-positive cells and then cell death was analyzed by propidium iodide staining and flow cytometric analysis 30 h after selection, as described in MATERIALS AND METHODS. The percentage of apoptosis was normalized for the percentage of CD14-positive cells in the enriched population (~80% routinely). Data shown represent the means ± SE of at least three experiments.

vector controls (Figure 1). In the experiment shown, ~66% of the cells expressing Cdc42(Q61L) were lost. These data suggested the possibility that expression of the constitutively active form of Cdc42 in Jurkat cells induced a regulated cell death response.

Because the dead cells observed in these experiments were those expressing the highest levels of Cdc42(Q61L), we further evaluated the remaining cells for 24 h. As shown in Figure 2, the cells expressing Cdc42(Q61L) at lower levels underwent a cell death response. This death program included nuclear condensation and DNA fragmentation accompanied by morphological changes, including shrinkage of the cell body and the formation of membrane blebs (Figure 3), all characteristics of an apoptotic response. Expression of wild type Cdc42 also induced an apoptotic response, generally of lesser magnitude than was observed with the constitutively active mutant, while a GDP-bound inactive form of Cdc42 did not (Figure 2). These results suggested that apoptosis was a result of downstream signaling by active Cdc42. Expression of constitutively active Rac1(Q61L) also induced apoptosis to some degree. In contrast, expression of either active RhoA(Q63L) or a constitutively active form of Ras did not induce cell death under these conditions. These data indicate that Cdc42, and to a lesser degree Rac1, activity specifically induces the apoptotic cell death program in Jurkat cells.

Signaling Mechanism Used by Cdc42 to Induce Apoptosis

We investigated the mechanism used by Cdc42 to induce apoptotic responses in the Jurkat cells. It has been shown in neurons, fibroblasts, and cells of myeloid lineage that induction of apoptosis by various types of cell "stress" is accompanied by activation of the JNK pathway (Westwick *et al.*, 1995; Cahill *et al.*, 1996; Chen *et al.*, 1996b; Cosulich and Clarke, 1996; Frisch *et al.*, 1996; Verheji *et al.*, 1996; Wilson *et al.*, 1996; Zanke *et al.*, 1996). The JNK pathway seems to generate "signals" that determine whether the cells undergo an apoptotic response (Ham *et al.*, 1995; Xia *et al.*, 1995; Chen *et al.*, 1996a,b). Recently, a number of laboratories have shown that the GTPases Rac and Cdc42 are able to stimulate the kinase cascade that regulates JNK activity (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Zhang *et al.*, 1995). We therefore evaluated the possibility that apoptosis induced by Cdc42 involved components of the JNK kinase cascade. Expression of activated mutants of both Cdc42 and Rac1 in Jurkat cells stimulated activity of the JNK pathway, as determined by direct analysis of c-Jun phosphorylation by JNK (Figure 4A). In contrast, no increases in JNK activity were observed with either vector controls or in the presence of active RhoA(Q63L). Cdc42(Q61L) was more effective in stimulating JNK activity, causing both earlier and greater increases in activity than did Rac1(Q61L), consistent with their relative ability to induce an apoptotic response.

MEKK and JNKK, respectively, are the kinases that act just upstream of JNK to regulate its activity (see Figure 9). Jurkat cells were transiently transfected with Cdc42(Q61L) in the presence or absence of kinase-inactive forms of MEKK and JNKK. The kinase-inactive forms of these proteins have been shown to inhibit JNK activation in response to upstream stimuli, presumably by binding to and preventing activation of downstream components in the cascade (Minden *et al.*, 1994; Lin *et al.*, 1995). As shown in Figure 5, expression of both dominant negative MEKK and dominant negative JNKK effectively blocked apoptosis induced by Cdc42(Q61L). Neither construct had any effect on the cells when expressed alone. The inhibitory effect on apoptosis induced by Cdc42(Q61L) was paralleled by the ability of dominant negative MEKK and JNKK to block Cdc 42-induced JNK activity (Figure 4B). These results indicate that an intact signaling pathway to JNK is required for an apoptotic response to Cdc42.

If Cdc42 induces apoptosis via the kinase cascade leading to JNK activation, then stimulation of this

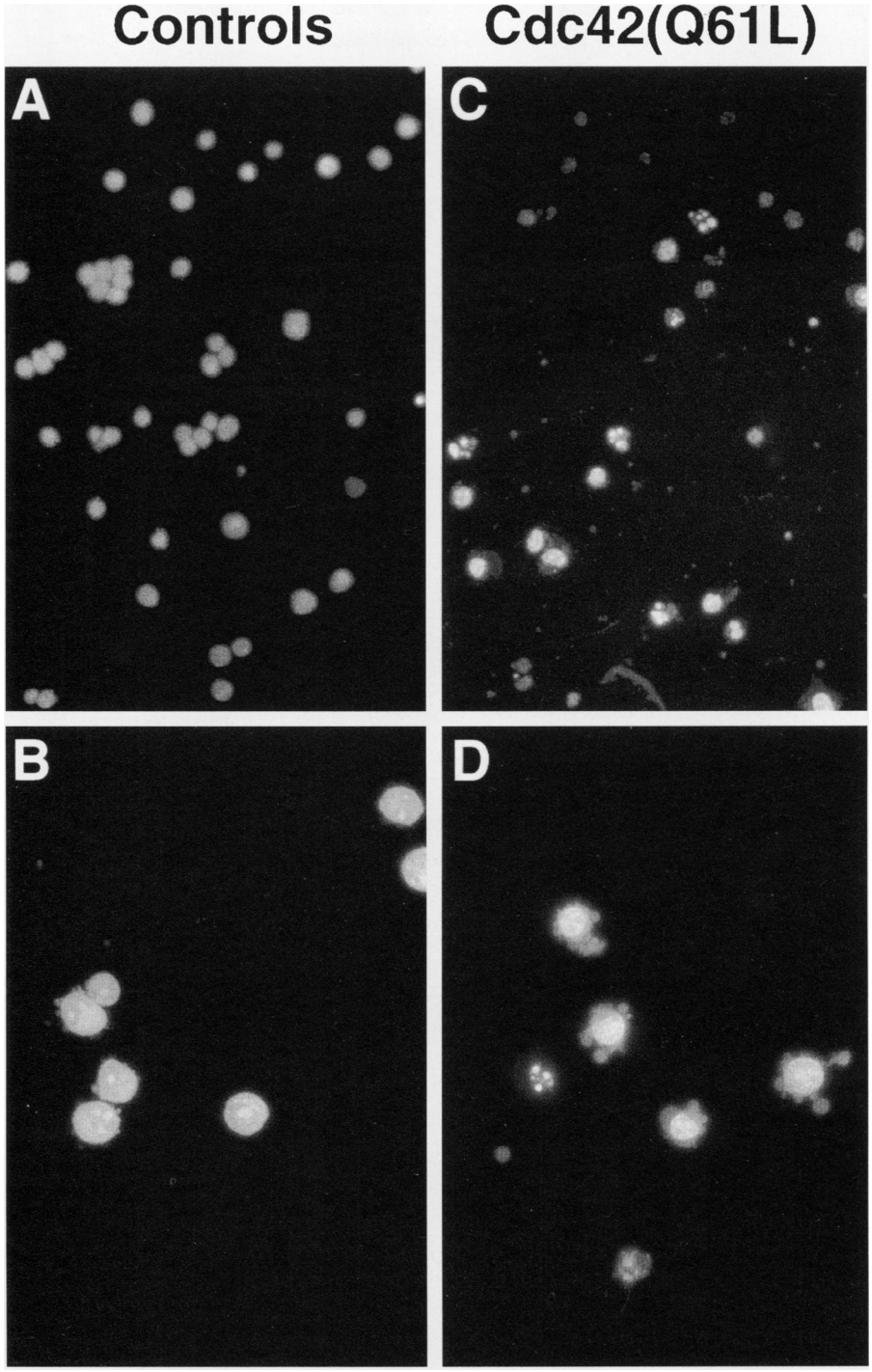

Figure 3. Nuclear fragmentation and morphological changes induced by Cdc42. Jurkat T cells transfected with control vector (A, 20×; B, 40×) or Cdc42(Q61L) (C, 20×; D, 40×) were stained with ethidium bromide and acridine orange for microscopic analysis of nuclear and cellular morphology, as described in MATERIALS AND METHODS. Membrane blebbing and nuclear condensation were observed in the Cdc42-induced apoptotic cells.

pathway using activated components at any point in the cascade should also elicit an apoptotic response. As shown in Figure 6A, we observed that a constitutively active form of MEKK ($MEKK^+$) effectively induced apoptosis to a comparable degree as did Cdc42(Q61L). We have also observed that expression of an additional component downstream of Cdc42, a constitutively active form of PAK2, induces the apoptotic response. In contrast to $MEKK^+$ and PAK2, expression of a constitutively active form of Raf to stimulate the ERK MAP kinase signaling pathway did not induce cell death. Expression of wild type JNK

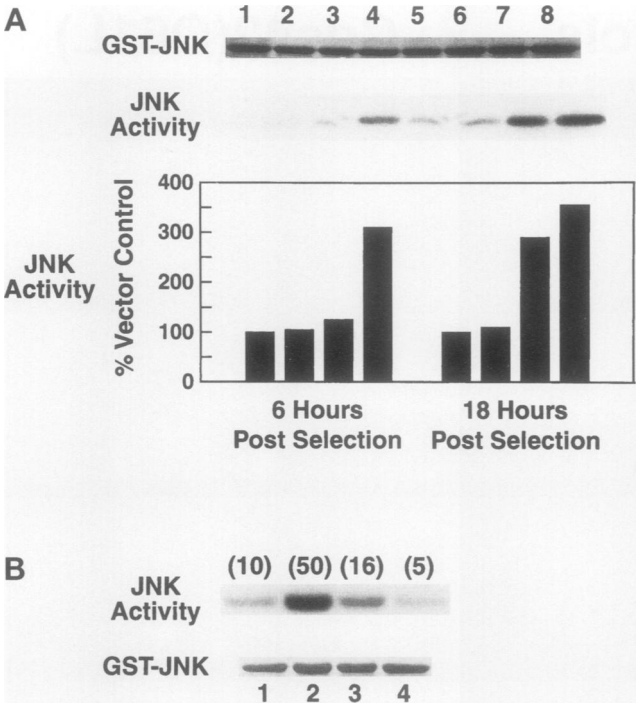

Figure 4. Cdc42(Q61L) induces JNK activation in Jurkat T cells. Phosphorylation of the substrate GST-c-Jun by JNK (c-Jun activity) was determined by solid-phase assay, as described in MATERIALS AND METHODS, in Jurkat cells transfected and selected for expression of the indicated cDNAs. (A) Lanes 1 and 5, vector control pcDNA3; lanes 2 and 6, pCMV5/RhoA(Q63L); lanes 3 and 7, pCMV5/Rac1(Q61L); lanes 4 and 8, pcDNA3/Cdc42(Q61L). Lanes 1–4 were assayed 6 h after selection and lanes 5–8 at 18 h after selection. The upper panel in A shows protein staining with Coomassie blue indicating that the level of substrate was identical in each assay. Actual JNK activity is shown by autoradiography (middle panel) or after quantitation of the same experiment by a PhosphoImager (bottom panel). The data shown were normalized to the vector (100%) and are the average of two similar experiments. (B) Lane 1, vector control pcDNA3; lane 2, Cdc42(Q61L) alone; lane 3, Cdc42(Q61L) plus dominant negative MEKK(K432M); lane 4, Cdc42(Q61L) plus dominant negative JNKK(K116R). JNK activity was assessed at 6 h after transfection and assay conditions were as described above. Numbers in parentheses indicate the relative level of activity quantified by a PhosphoImager.

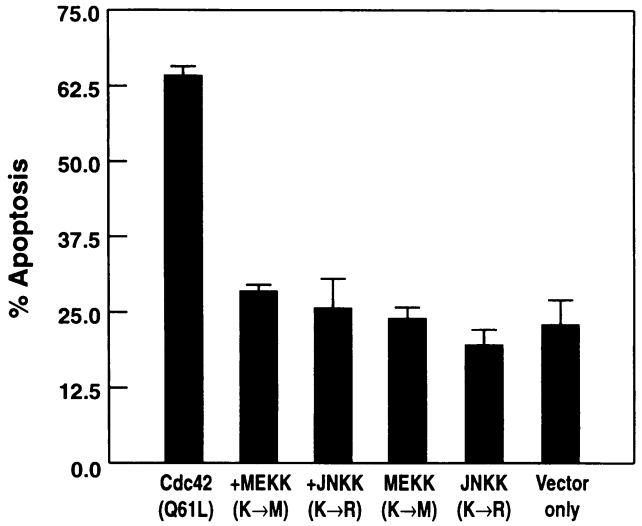

Figure 5. Inhibition of Cdc42(Q61L)-induced apoptosis by dominant negative MEKK and JNKK mutants. Jurkat T cells were cotransfected with CD14 and control or Cdc42(Q61L) expression vectors in the presence (+) or absence of dominant negative MEKK(K432 M) or JNKK(K116R) expression vectors at 1:6:9 cDNA ratios. In each transfection, the total amount of cDNA was supplemented to the same level with control vector. Cells were selected and analyzed for apoptosis as described for Figure 2. Results shown are the means ± SE for at least three experiments.

itself caused a slight increase in the number of apoptotic cells, whereas expression of the related mitogen-activated protein (MAP) kinase p38 had no effect. We determined that each of these kinase constructs was functional by measuring the activation of the Elk transcription factor using a luciferase reporter assay (Figure 6B).

Cdc42-induced Apoptosis Is Blocked by ICE Protease Inhibitors

Apoptotic responses in T cells are known to involve the activity of proteases of the extended ICE family, and apoptosis in these cells is characteristically blocked by inhibitors of CPP32/caspase-3, as is cleavage of D4 GDI (Na *et al.*, 1996). To determine whether Cdc42 initiated an apoptotic signal that was downstream from and independent of the action of ICE proteases, we evaluated the effect of ICE inhibitors on the apoptotic responses induced by Cdc42(Q61L). The DEVD-CHO peptide effectively inhibits CPP32/caspase-3 and apoptosis (Nicholson *et al.*, 1995). Cell death induced by Cdc42 was effectively reduced by this peptide as well (Figure 7). In contrast to DEVD-CHO, the YVAD-CMK peptide, which is less active toward CPP32 and more inhibitory for ICE protease (Lazebnik *et al.*, 1994), was less effective in blocking the apoptotic response to Cdc42(Q61L) at the concentrations used. This is consistent with the relative effects of these inhibitors on apoptosis induced by Fas ligation and other stimuli in Jurkat cells (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Na *et al.*, 1996). The death response to MEKK$^+$ was also blocked by ICE protease inhibitors (our unpublished observations).

CrmA is a viral protein that blocks apoptosis by inhibiting ICE protease action (Tewari and Dixit, 1995). Expression of CrmA in Jurkat cells substantially, although not completely, attenuated the level of cell death induced by Cdc42(Q61L) (Figure 7). The incomplete inhibition may reflect the level of CrmA expression achieved, but cannot be differentiated from an actual partial effect on the response. Bcl2 also acts as a negative regulator of cell death (Reed, 1994). Although its exact

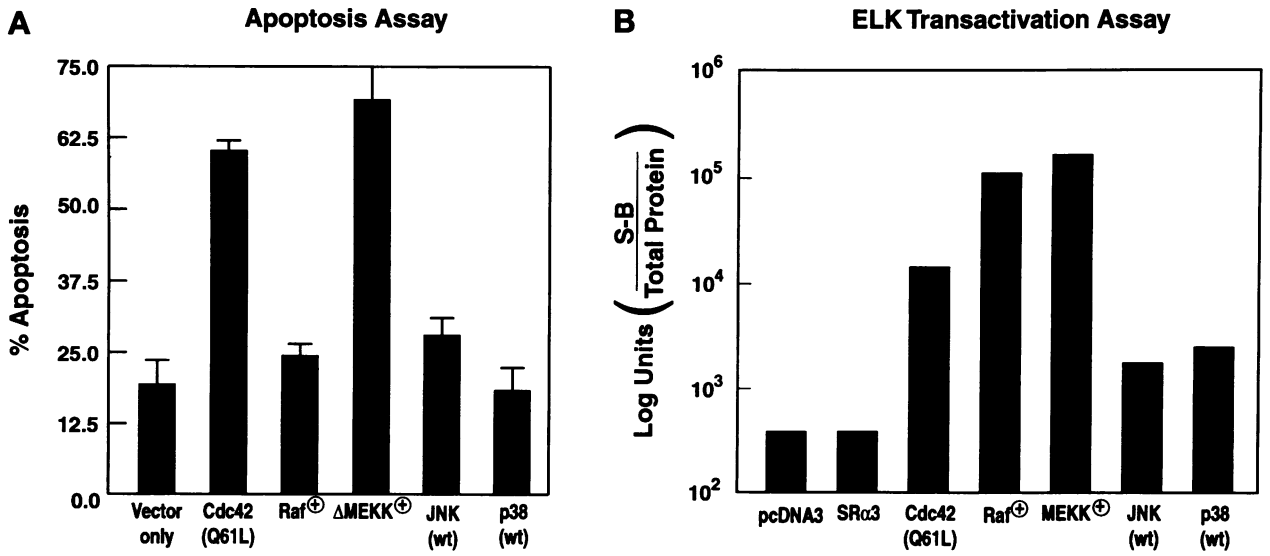

Figure 6. Induction of apoptosis of Jurkat T cells by kinases. (A) Jurkat T cells were cotransfected with expression vectors for CD14, Cdc42(Q61L), or the indicated kinase constructs at a 1:8 cDNA ratio. Transfected cells were selected and analyzed for cell death as described in Figure 2. (B) The activity of the expressed constructs was verified by analysis of ELK transcription, as described in MATERIALS AND METHODS. Raf$^+$ and MEKK$^+$ represent the activated forms of these kinases.

mechanism(s) of action are unknown, it also seems to be able to block the action of certain ICE proteases (Armstrong *et al.*, 1996; Chinnaiyan *et al.*, 1996). We observed that expression of Bcl2 inhibited the cell death response to Cdc42(Q61L). Again, this inhibition was not complete.

We examined cleavage of D4 GDI (Na *et al.*, 1996) when Jurkat cell death was induced by Cdc42(Q61L) and observed the formation of the 23 kDa CPP32-mediated cleavage product. Such data strongly support the involvement of CPP32/caspase-3 and/or other related proteases as downstream elements of the signaling pathway induced by Cdc42.

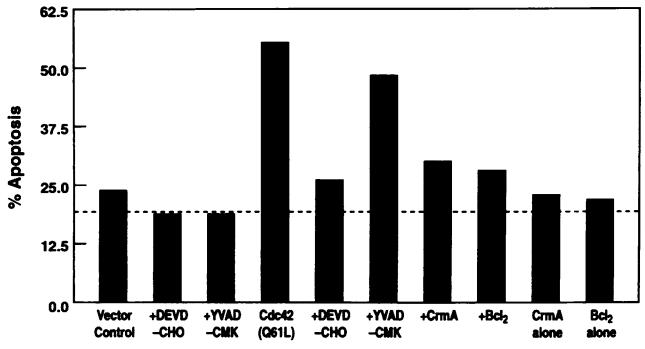

Figure 7. Inhibition of Cdc42(Q61L)-induced apoptosis by ICE protease inhibitors. Jurkat T cells were cotransfected with CD14 and either vector control or pcDNA3/Cdc42(Q61L) in the presence or absence of the CrmA or Bcl2 expression plasmids at a cDNA ratio of 1:6:9. In each transfection, the total cDNA was supplemented to the same level with control vector. Cells were selected after 14 h; where indicated, the cells were incubated with or without 100 μg/ml Ac-YVAD-CMK or Ac-DEVD-CHO for an additional 30 h. Apoptosis was analyzed as described in MATERIALS AND METHODS.

Cdc42 Modifies the Normal Morphological Program in Apoptotic Cells

Cells undergoing apoptotic death progress through a very characteristic series of morphological changes (Ellis *et al.*, 1991; Martin *et al.*, 1994; Kerr, 1995). Because of their large nuclei and limited cytoskeleton, these changes are difficult to observe in the Jurkat T cells. We therefore turned to another Fas-mediated apoptotic system, that of CHO (Chinese hamster ovary) cell killing by cytotoxic T lymphocytes, where the large well-spread target CHO cell could be readily observed by DIC microscopic imaging (Hahn *et al.*, 1994). Examination of the morphological changes accompanying apoptosis in response to killing of targeted CHO cells by cytotoxic T lymphocytes reveals a series of changes typical of the apoptotic death response. The cells undergo a pronounced contraction of the cell body, beginning with membrane edge retraction and followed by rapid shrinkage and cell rounding (Figure 8, A1,2). This is accompanied at the early stages of contraction by the appearance of membrane blebs (Figure 8, A2). Finally, the cell undergoes further contraction, accompanied by extensive membrane blebbing, to form apoptotic bodies for eventual disposal by phagocytosis (Figure 8, A3).

Although expression of constitutively active Cdc42(Q61L) does not appear itself to induce cell death in CHO cells in the presence of serum, the morphological

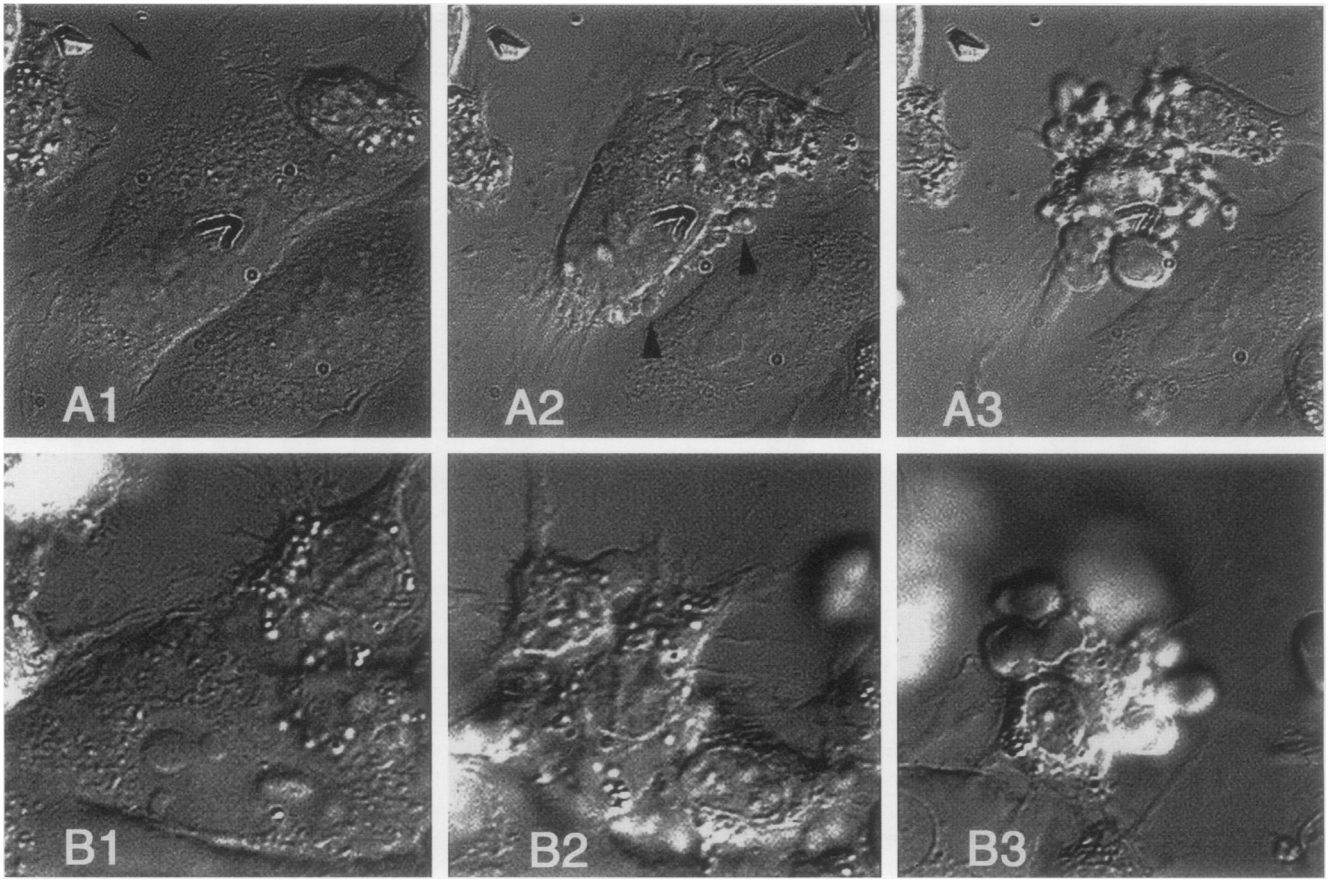

Figure 8. Activated Cdc42 modifies the normal morphological sequence in apoptotic cells. The images illustrate the effects of Cdc42Q61L on target cells undergoing cytotoxic T lymphocyte (CTL)-induced apoptosis. In series A, target CHO cells were transfected with wild-type Cdc42. Frame A1 shows a large CHO cell being approached by two smaller CTLs to its right and left. The arrow indicates the edge of the target cell, which is somewhat difficult to see because the cell is very thin. Frame A2 shows small blebs forming at the cell edge (arrowheads) during gradual contraction of the cell body. In frame A3 the cell has collapsed completely and is producing large blebs. A CTL to the upper right of the target cell remains attached throughout the image series. This behavior is typical of nontransfected cells or those transfected with low levels of wild-type Cdc42. The series of images B1–B3 illustrate the altered apoptotic changes produced by transfection with Cdc42Q61L mutant. Frame B1 shows a CTL attached to the upper right of a large CHO cell. Here, the CHO cell collapses very rapidly without blebbing. Frame B2, immediately after rapid contraction, shows that the cell is no longer visible, and the area it covered previously is occupied by CTLs, which converged on the cell fragments during contraction. In the final frame, cell remnants and large blebs are visible. A1, 0 min; A2, 30 min; A3, 44 min; B1, 0 min; B2, 25 min; and B3, 43 min). *Note*: The v-shaped object in frames A1–A3 represents debris on the camera lens.

program is dramatically altered when the same cells are expressing low levels of Cdc42(Q61L). At these levels of expression, Cdc42(Q61L) has no obvious effect by itself on the morphology of the CHO cells (Figure 8, B1). However, when killing is induced in the target cells by contact with the cytotoxic T lymphocytes, the initial contraction phase observed is accelerated, preceded by only slight rounding of the apoptotic cell (Figure 8, B2). The dying cell suddenly changes in appearance, leaving only distorted remnants attached to the substratum. Membrane blebbing is now apparent primarily in the final phase of cell death, and the blebs are somewhat larger in size. The dead cell finally forms a mass of blebbing cell debris (Figure 8, B3).

DISCUSSION

Cdc42 as an Inducer of Apoptosis

The common features of cellular apoptosis include contraction of the cell body accompanied by membrane blebbing, nuclear condensation, and DNA fragmentation, and the externalization of phosphatidylserine on the cell surface to enhance phagocytic uptake of the resulting cellular fragments or apoptotic bodies. This complex series of events implies that apoptosis is not a simple process, but is likely to require diverse signals to multiple molecular targets to coordinate the process of cell death. The results presented here establish for the first time the ability of the small GTPase Cdc42 to initiate signals leading to both nuclear deg-

radation and cytoskeletal reorganization within the context of an apoptotic response.

We have shown that Cdc42 stimulates apoptosis in Jurkat T cells through a JNK signal transduction cascade (Figure 9). Cdc42-induced cell death can be blocked by coexpression with dominant negative mutants for MEKK and JNKK. In contrast, expression of a dominant active MEKK can also lead to Jurkat T cell apoptosis. Active forms of MEKK have also been reported to induce cell death when expressed in fibroblasts (Johnson *et al.*, 1996) or PC12 cells (Xia *et al.*, 1995). Our results are consistent with the roles of Cdc42 and Rac in regulating JNK and p38 MAP kinase signaling cascades in a variety of cells (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Zhang *et al.*, 1995), and with the important role ascribed to JNK kinase activity in apoptotic responses in neuronal cells and a variety of other cell types (Ham *et al.*, 1995; Westwick *et al.*, 1995; Xia *et al.*, 1995; Cahill *et al.*, 1996; Chen *et al.*, 1996a,b; Frisch *et al.*, 1996; Verheji *et al.*, 1996; Wilson *et al.*, 1996; Zanke *et al.*, 1996). It has been observed that various apoptotic stimuli, such as Fas ligand, TNF-α, ceramides, serum, or growth factor withdrawal, detachment of cells from the extracellular matrix, and UV-radiation are able to concomitantly stimulate JNK activity in cells. Additionally, activation of the Rac GTPase has been observed in response to Fas-induced cell death (Gulbins *et al.*, 1996). We have attempted to block the cell death induced by Fas or ceramides in Jurkat cells by expression of dominant negative Cdc42(T17N) mutant, but have been unable to achieve more than a modest decrease in the response to ceramides. This may due to the inability to attain high expression levels of Cdc42(T17N) in the Jurkat cells—the dominant negative effect relies upon effective mass competition of the mutant GTPase with endogenous Cdc42 guanine nucleotide exchange factors. It is also possible that the Rac present and activated in the stimulated cells may be able to bypass the need for active Cdc42.

Although JNK activation does not seem to play a major role in cell death induced by the Fas receptor (Lenczowski *et al.*, 1997; Rudel and Bokoch, unpublished observations) and there are contrary reports about the role of JNK in TNF-α-induced apoptosis, JNK activity clearly seems to be important in stress-induced apoptotic responses (Ham *et al.*, 1995; Westwick *et al.*, 1995; Xia *et al.*, 1995; Cahill *et al.*, 1996; Chen *et al.*, 1996a,b; Frisch *et al.*, 1996; Verheji *et al.*, 1996; Wilson *et al.*, 1996; Zanke *et al.*, 1996). Indeed, death induced by such stimuli is relatively slow when compared with death induced by members of the death receptor (Fas/TNF) family, the former taking more than 24 h to occur versus less than 1–6 h for Fas. This is very similar to the death response induced by activated Cdc42, which required 24–36 h of protein expression for maximum death to occur when expressed at modest levels. (The highly expressing cells were likely to have died earlier; see Figure 1.) Additionally, Cdc42 is known to be involved in growth factor signaling (Nobes and Hall, 1995; Olson *et al.*, 1996) and recent work suggests Cdc42 is a component of cell adhesion signaling via integrins (Schwartz and Bokoch, unpublished observations). The loss of such growth factor and adhesion signals can induce apoptosis in certain cell types over a similarly extended time frame of 24–48 h.

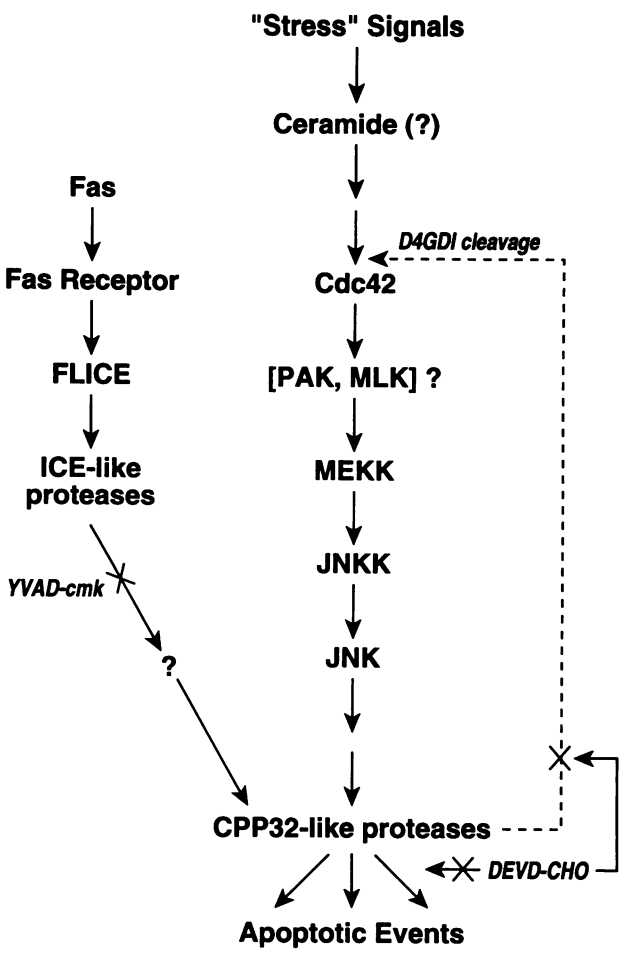

Figure 9. Model of Cdc42-regulated apoptotic signaling. Cdc42 induces apoptosis through a JNK-dependent signaling pathway. This apoptotic signaling induces caspase activity which can cleave D4 GDI and release more Cdc42 in a positive feedback loop. This pathway is distinct from that used by the Fas/CD95 death receptor, but may be involved in stress-, growth factor withdrawal-, or loss of adhesive signaling-induced death signaling.

Cdc42 Acts Upstream of Caspases

Cell death induced by Cdc42 was inhibited by ICE protease inhibitory peptides and CrmA (Figure 7). These data indicate that the activation of the JNK

pathway by Cdc42 leads through some as yet unknown mechanism to caspace activation. Cdc42 is thus an upstream stimulus for cell death and must activate caspases for the death response to occur. The sensitivity to DEVD peptides and relative insensitivity to YVAD peptides suggests that the Cdc42/JNK pathway may interact at a point below the YVAD-sensitive ICE-like proteases and above the DEVD-sensitive CPP32-like proteases (Figure 9). Caspase activation mediated by JNK has also been reported recently in DNA-damaging drug-induced apoptosis (Seimiya *et al.*, 1997). The possible release of Cdc42 and related Rho family GTPases from D4 GDI after cleavage by CPP32 (caspase-3) may serve as a positive feedback mechanism to increase the amount of active Cdc42 and, ultimately, CPP32 (Figure 9). It should be noted that JNK activation as a result of stimulation of the caspase cascade has also been observed (Cahill *et al.*, 1996; Juo *et al.*, 1997; Lenczowski *et al.*, 1997).

A Role for Cdc42 in Coordinating Morphological Changes during Apoptosis?

When we examined the morphological changes occurring in target CHO cells during cell death induced by cytotoxic T lymphocytes, we found that constitutive active Cdc42(Q61L) dramatically modified the normal sequence of these events. Because this Cdc42 mutant remains in an active form continuously, it is likely to alter the normal balance of signals controlling and coordinating cytoskeletal behavior. These data suggest that Cdc42, consistent with its ability to modulate complex cytoskeletal dynamics during cell growth, motility, and differentiation (Nobes and Hall, 1995), might also play an important role in controlling cytoskeletal processes in the dying cell. Cdc42 and related Rho family GTPases may thus provide a means to coordinate cellular death signals with cytoskeletal processes important for final packaging of the apoptotic cell for disposal by phagocytic mechanisms.

In conclusion, we have described the ability of the Rho family GTPase Cdc42 to effectively induce and modulate apoptotic responses in Jurkat T cells. It is of interest that the gene defective in Aarskog-Scott syndrome or faciogenital dysplasia, termed FGD1, has been shown to be a guanine nucleotide exchange factor for Cdc42 (Olson *et al.*, 1996; Zheng *et al.*, 1996). This disease is characterized by impaired growth and both facial and urogenital malformations, and it is believed that the normal development of the skeletal and urogenital systems are affected. Although not demonstrated yet, this could very possibly be a manifestation of Cdc42-induced apoptosis in these tissues during development.

We have also observed that the expression of an activated form of Rac2 (Rac2Q61L) under the control of a thymus-specific lck promoter results in a dramatic arrest in thymic development (Aichinger, Xu, Peterson, and Bokoch, unpublished observations). There was a marked decrease in the number of double positive ($CD4^+/CD8^+$) cells in the thymus associated with a reduction in mature peripheral T cells. This effect was not observed in animals expressing an inactive Rac2T17N mutant. These findings are also consistent with Rac2Q61L-induced apoptosis during early thymic development. More detailed analysis of the role of Rho GTPases in controlling cytoskeletal dynamics and apoptotic signaling pathways will be a goal of future work from our laboratories.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Yan Wang, MD, and Chester Chamberlin, BS. We are grateful to Hanna Lewicki and M.B.A. Oldstone for supplying cytotoxic T lymphocytes for these studies. This work was supported by National Institutes of Health grants to G.M.B. and K.M.H., and American Heart Association (California Affiliate) and National Arthritis Foundation fellowships to T.H.C. This is publication no. 10748-IMM from The Scripps Research Institute.

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