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*Advances in Brief*

# Requirement of p34$^{cdc2}$ Kinase for Apoptosis Mediated by the Fas/APO-1 Receptor and Interleukin 1β-converting Enzyme-related Proteases[1]

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## Abstract

**The induction of apoptosis by the Fas/APO-1 receptor is important for T-cell-mediated cytotoxicity and down-regulation of immune responses. Binding of Fas ligand to the Fas/APO-1 receptor transduces an apoptotic signal that requires activation of interleukin 1β-converting enzyme (ICE) and CPP32β, members of a family of cysteine proteases that are evolutionarily conserved determinants of cell death. We report here that Fas/APO-1-triggered apoptosis involves ICE-mediated activation of p34$^{cdc2}$ kinase. Ligation of the Fas receptor resulted in the rapid stimulation of ICE proteolytic activity and activation of p34$^{cdc2}$ kinase. Specific tetrapeptide inhibitors of ICE (Acetyl-Tyr-Val-Ala-Asp-chloromethylketone) or CPP32β (Acetyl-Asp-Glu-Val-Asp-aldehyde) prevented the anti-Fas antibody-mediated activation of p34$^{cdc2}$ and inhibited apoptosis. Inhibition of p34$^{cdc2}$ activity by transient overexpression of a dominant-negative cdc2 construct or human WEE1 kinase inhibited Fas-mediated apoptosis. These results suggest that activation of p34$^{cdc2}$ kinase is a critical determinant of cell death mediated by Fas and ICE family proteases.**

## Introduction

The ability of the Fas/APO-1/CD95 receptor to trigger apoptosis is dependent on an evolutionarily conserved intracellular "death domain" that mediates the ligand-dependent recruitment of FADD/MORT1[3] (1–3). The formation of Fas/FADD complexes initiates an apoptotic signal that requires the sequential activation of members of the ICE family of cysteine proteases, including FLICE/MACH, ICE, and CPP32β (Apopain/Yama; Refs. 3–6). Although the mechanism by which Fas ligation activates the ICE-related proteolytic cascade has been well defined, the vital substrate(s) and downstream effectors involved in the induction of apoptosis by the ICE/CED-3 family of cysteine proteases have yet to be elucidated. ICE-related proteases are distinguished by an unusual substrate specificity (cleavage at aspartic acid-amino acid bonds; Refs. 7–10) that is shared only by granzyme-B, a serine protease responsible for apoptosis induced by CTLs (11). Induction of apoptosis by granzyme-B, a direct activator of CPP32β (12), has been shown to require activation of p34$^{cdc2}$ kinase, a universal determinant of entry into mitosis (13). Cell viability requires regulatory mechanisms that restrain the activity of the Cdc2-cyclin B complex (mitosis promoting factor) until completion of DNA replication and repair. Unscheduled activation of p34$^{cdc2}$ kinase results in lethal phenotypes (termed "mitotic catastrophes"), which exhibit hypercondensed chromatin and chromosomal fragmentation reminiscent of the characteristic morphological changes observed during apoptosis (14). Because Fas ligation also stimulates ICE and CPP32β-related proteolytic activity (3–6), we investigated whether Fas-induced apoptosis involves ICE-mediated activation of p34$^{cdc2}$ kinase.

## Materials and Methods

**Anti-Fas Antibody Mediated Apoptosis.** Jurkat cells were synchronized in late G1 with aphidocolin (1 μg/ml, 16 h) and released into S phase by washing (×3) followed by addition of anti-Fas antibody (1 μg/ml). Fas-treated cells and untreated controls were harvested at 15–30-min intervals for Western analyses and assessment of H1 kinase activity of p34$^{cdc2}$ immunoprecipitates.

**Inhibition of Anti-Fas Antibody Mediated Apoptosis and p34$^{cdc2}$ Kinase Activation by ICE/CPP32β-related Protease Inhibitors.** G1-synchronized Jurkat cells (5 × 10$^4$/ml) were preincubated (3 h) with inhibitors of ICE- or CPP32β-related proteases [Ac-YVAD-cmk (BACHEM, Torrance, CA; 600 uM) or Ac-DEVD-CHO (BACHEM; 300 uM)] or without the inhibitors, and then treated with 1.0 μg/ml antihuman Fas antibody (United Biomedical, Inc., Lake Placid, NY) in the absence or presence of the inhibitors. Cells were analyzed for p34$^{cdc2}$ kinase activity after 2 h and assessed for apoptosis 6 h later by Hoechst staining of nuclei. The percentage viability was calculated by scoring at least 200 cells in each group in two different experiments.

**Immunoblotting and p34$^{cdc2}$ Kinase Assay.** Cells were lysed by addition of 100 μl of ice-cold lysis buffer [10 mM Tris (pH 7.4), 50 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 0.5% NP40] containing 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM β-glycerophosphate. The lysates were centrifuged at 15,000 × *g* for 10 min at 4°C, and total protein was quantified by bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) with albumin as standard. p34$^{cdc2}$ immunoprecipitation was performed using 100 μg of total cell lysate and protein A-Sepharose, as described (13). The resultant immunoprecipitates were analyzed for histone H1 kinase activity using H1 buffer [50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, and 50 μM ATP] and 10 μCi [$\gamma^{32}$P]ATP (>3000 Ci/mM), followed by 12.5% PAGE and autoradiographic assay of histone H1 phosphorylation. p34$^{cdc2}$ immunoprecipitates were also subjected to 12.5% SDS-PAGE, immunoblotted, probed with an antibody p34$^{cdc2}$, and developed with horseradish peroxidase-conjugated antibody to mouse IgG (for 4G10) or horseradish peroxidase-conjugated goat antibody to rabbit IgG (for p34$^{cdc2}$). Total cellular protein (100 μg) was simultaneously subjected to 12.5% SDS-PAGE, immunoblotted, and probed with anti-p34$^{cdc2}$ or anti-cyclin B1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). All immunoblots were developed with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

**Transfection of Dominant-negative cdc2 (cdc2dn-HA) or Wee1 Kinase (WEE1) into Jurkat Cells.** Jurkat cells in exponential phase were maintained in RPMI 1640 supplemented with 10% FBS at a maximum density of less than 7.5 × 10$^4$ cells/ml during the 12 h before transfection. Transfections were performed in 35-mm wells using 4 × 10$^5$ cells in 250 μl of serum-free medium (Opti-MEM) supplemented with DMRIE-C reagent (10 μl/ml; Life Technologies, Gaithersburg, MD) and the following expression plasmids: cdc2dn-HA (encoding dominant-negative cdc2 constructed in the CMV-neo-Bam vector (provided by Dr. Ed Harlow; Ref. 15), WEE1 (Wee1 cDNA in the correct orientation with respect to the α-globin gene promoter), or WEE1-rev (Wee1 cDNA in the reverse orientation), constructed in the mammalian expression vector pCMUIV (provided by Dr. Paul Russell; Ref. 16). For each well, 400 ng

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[3] The abbreviations used are: FADD, Fas-associated protein with death domain; ICE, interleukin 1β-converting enzyme; FBS, fetal bovine serum; β-Gal, β-galactosidase; CED, *Caenorhabditis elegans* death; Ac-YVAD-cmk, Acetyl-Tyr-Val-Ala-Asp-chloromethylketone; Ac-DEVD-CHO, Acetyl-Asp-Glu-Val-Asp-aldehyde.

of the lacZ eukaryotic expression construct CMV-β-Gal (containing the *Escherichia coli* β-Gal reporter gene) and 1200 ng of the cdc2dn-HA, WEE1, or WEE1-rev expression plasmid were used (reporter:gene = 1:3, total DNA = 1600 ng in 100 μl). Four h later, cells were supplemented with growth media (RPMI + 10% FBS, 1 mg/ml phytohemagglutinin, 1 μg/ml phorbol 12-myristate 13-acetate) and incubated for 12 h at 37°C. The cells were washed three times with RPMI (+ 10% FBS) and treated with anti-Fas antibody (1 μg/ml) for 12 h. To detect reporter gene expression, cells were fixed with 2% paraformaldehyde containing 1% glutaraldehyde for 5 min, rinsed with PBS, and stained for 12 h in X-Gal buffer containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indoxyl-β-Galactoside.

**Assessment of Apoptosis in Transfected Cells.** X-Gal-stained slides were incubated with Hoechst 33342 dye (5 mM for 1 min), and X-Gal-positive cells were analyzed by fluorescence microscopy for evidence of apoptosis (condensed, fragmented chromatin). The percentage of apoptotic cells among the total number of X-Gal-positive (blue) cells was calculated by scoring at least 200 transfected cells from each group in two different experiments. At 12 h after transfections, cells were stained with a rabbit anti-β-Gal antibody (Cappel, Durham, NC) followed by a FITC-conjugated anti-rabbit IgG secondary antibody and labeled with propidium iodide; β-Gal-positive cells from each group were analyzed for cell cycle distribution by flow cytometry.

## Results and Discussion

Treatment of aphidocolin-synchronized human Jurkat cells (T-cell leukemia) with antihuman Fas antibody resulted in the rapid activation of p34$^{cdc2}$ kinase within 90 min of exposure (Fig. 1*A*) followed by the induction of apoptosis within 6 h (Fig. 2*A*). Fas-mediated activation of p34$^{cdc2}$ also occurred in nonsynchronized cells, although the magnitude of the increase in histone H1 kinase activity was less than that observed after Fas ligation in synchronized cell populations (data not shown). The anti-Fas-mediated augmentation of p34$^{cdc2}$ kinase activity was not associated with any elevation of p34$^{cdc2}$ or cyclin B1 protein levels (Fig. 1*B*). Fas ligation also induced ICE-like proteolytic activity in Jurkat cells, which was detectable within 45 min

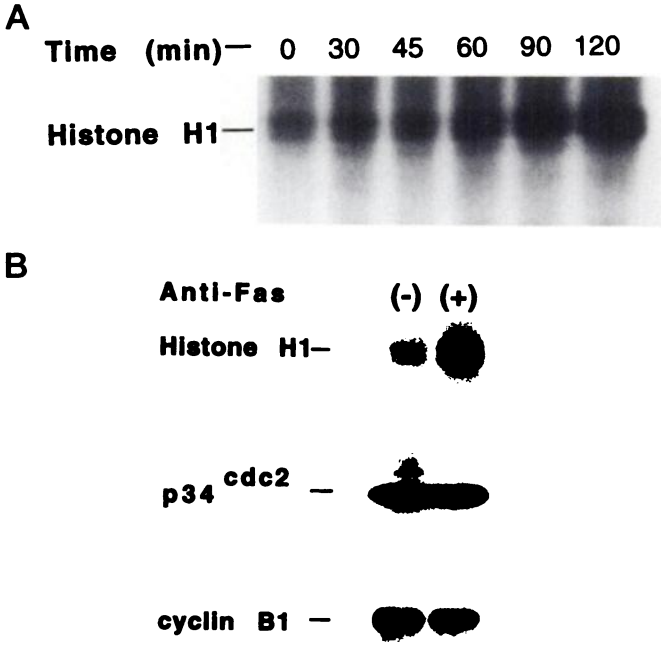


Fig. 1. Activation of p34$^{cdc2}$ kinase during anti-Fas antibody induced apoptosis. *A*, Synchronized populations of Jurkat cells were analyzed for p34$^{cdc2}$ kinase activity at the designated times following treatment with anti-Fas antibody (1 μg/ml). *B*, H1 kinase activity of p34$^{cdc2}$ immunoprecipitates and expression of p34$^{cdc2}$ and cyclin B1 proteins from cells treated with anti-Fas antibody (1 μg/ml) for 2 h (+) compared to untreated controls (−) at the same time point.

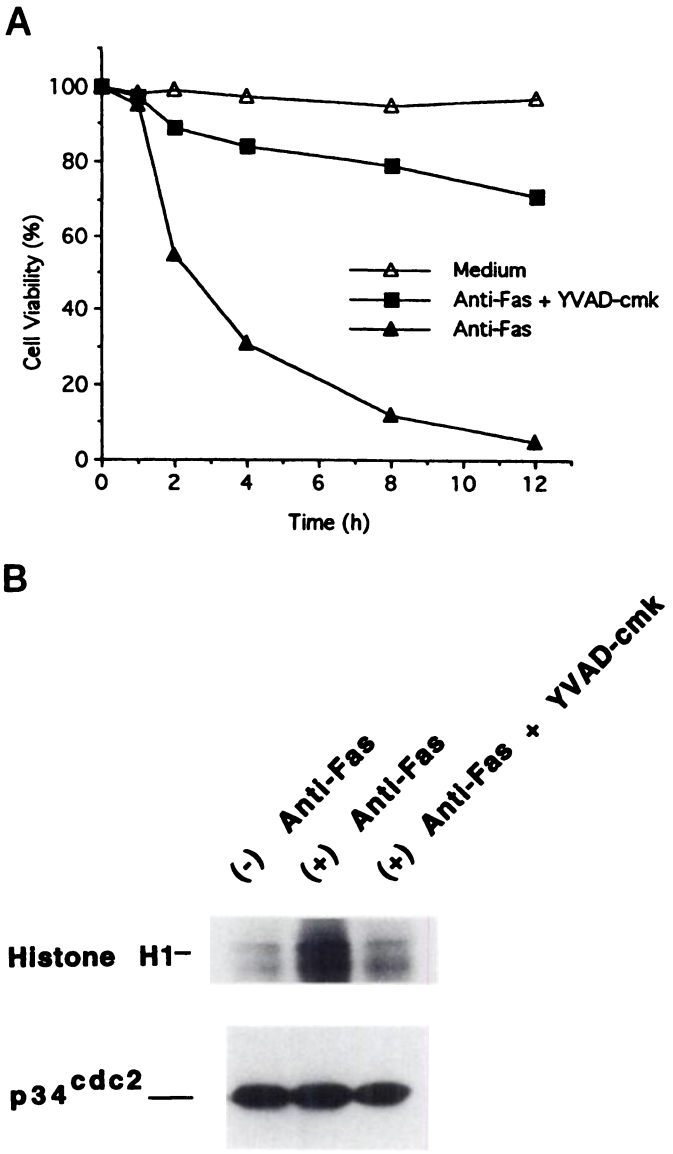


Fig. 2. *A*, effect of the ICE-related protease inhibitor, YVAD-cmk, on anti-Fas antibody-mediated apoptosis. Human Jurkat cells ($5 \times 10^4$) were preincubated (3 h) with YVAD-cmk (BACHEM; 600 μM) or without the ICE inhibitor, and then treated with 1.0 μg/ml antihuman Fas antibody (United Biomedical, Inc., Lake Placid, NY) in the absence or presence of YVAD-cmk. Cells were analyzed for apoptosis 6 h later by Hoechst staining of nuclei, and the percentage viability was calculated by scoring at least 200 cells in each group in two different experiments. *B*, inhibition of Fas-mediated activation of p34$^{cdc2}$ kinase by the tetrapeptide ICE inhibitor YVAD-cmk. Jurkat cells were synchronized in late G1 with aphidocolin (1 μg/ml), released into S phase by washing, and incubated with or without the ICE inhibitor YVAD-cmk for 2 h. The cells were then treated with (+) or without (−) anti-Fas antibody (1 μg/ml) and maintained in the presence or absence of YVAD-cmk for another 2 h before analysis of p34$^{cdc2}$ kinase activity.

by flow cytometric analysis of cleavage of a fluorogenic ICE substrate DABCYL-YVADAP-EDANS (data not shown). Inhibition of anti-Fas antibody-mediated apoptosis by the tetrapeptide ICE inhibitor Ac-YVAD-cmk (Fig. 2) or the CPP32β inhibitor Ac-DEVD-CHO prevented Fas-mediated activation of p34$^{cdc2}$ kinase. Other protease inhibitors, such as phenylmethylsulfonyl fluoride or leupeptin, had no effect on Fas-mediated activation of p34$^{cdc2}$ kinase or apoptosis.

To determine whether the activation of p34$^{cdc2}$ kinase is required for Fas-mediated apoptosis, p34$^{cdc2}$ activity was inhibited by expression of either dominant-negative cdc2 (cdc2dn-HA; Ref. 15) or human Wee1 kinase (16). The dominant-negative cdc2 construct contains a point mutation at residue 146 (substitution of Asn with Asp), which

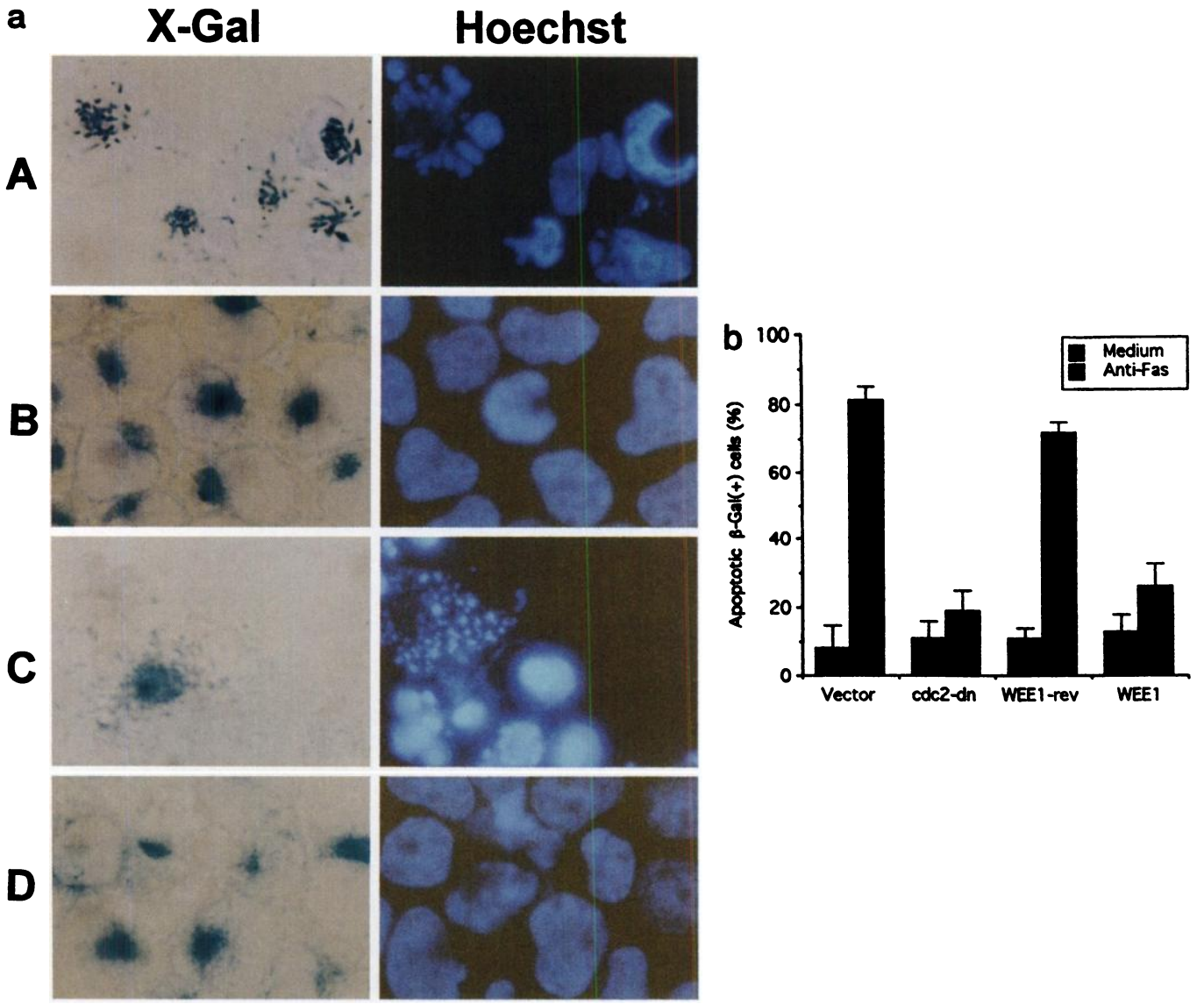


Fig. 3. *a*, effect of inhibition of p34$^{cdc2}$ kinase on anti-Fas antibody-mediated apoptosis. Jurkat cells were transfected with an expression vector encoding β-Gal (*A*) or cotransfected with β-Gal together with plasmids expressing dominant-negative cdc2 (cdc2dn-HA) (*B*), human Wee1 cDNA in the reverse orientation (WEE1-rev) (*C*), or human Wee1 cDNA (WEE1) (*D*). The cells were treated with anti-Fas antibody (1 μg/ml) 12 h after transfections and analyzed 12 h later for β-Gal expression by X-Gal staining (*X-Gal*) and apoptosis by Hoechst staining (*Hoechst*) of cell nuclei. The transfection efficiency of Jurkat cells using DMRIE reagent was >80%. *b*, effect of transient expression of dominant-negative cdc2 (cdc2dn-HA) or Wee1 kinase (WEE1) on Fas-induced apoptosis. The figure gives the percentage of apoptotic cells among the total number of X-Gal-positive (*blue*) cells in each group.

results in the loss of kinase activity without alteration of cyclin B binding ability (15). Previous studies have verified that overexpression of cdc2dn-HA results in a G2 cell cycle arrest (15). The *Wee1* gene encodes a nuclear kinase that prevents mitotic entry by tyrosine phosphorylation mediated inactivation of p34$^{cdc2}$ (16). Wee1 has been shown to protect mammalian cells from "mitotic catastrophes" induced by inappropriate activation of cytoplasmic p34$^{cdc2}$ (14). Jurkat cells were cotransfected with a β-Gal reporter gene plasmid (β-Gal) and either cdc2dn-HA or human Wee1 kinase (WEE1). Control cells were cotransfected with either WEE1-rev (the same vector encoding the Wee1 cDNA in the reverse orientation) and β-Gal, or the reporter gene alone. Cell cycle analysis of cells transfected with cdc2dn-HA or WEE1 showed a G2 arrest, whereas control cells transfected with WEE1-rev or β-Gal exhibited a normal cell cycle distribution (data not shown). Twelve h after transfection, cells were exposed to anti-Fas antibody (1 μg/ml), and β-Gal-positive cells were analyzed for apoptosis 12 h later. Hoechst staining revealed that treatment of Jurkat cells with anti-Fas antibody resulted in chromatin condensation and apoptotic nuclear fragmentation. Cells transfected with vector controls (WEE1-rev or β-Gal) remained susceptible to apoptosis induced by anti-Fas antibody (Fig. 3). In contrast, cdc2dn-HA- or WEE1-transfected cells exhibited a relative resistance to anti-Fas-induced apoptosis; blue-stained cells showed large uncondensed intact nuclei with a substantial reduction of apoptotic nuclei compared with cells transfected with vector controls (Fig. 3). To confirm that the inhibition of apoptosis conferred by cdc2dn-HA or WEE1 was due to inactivation of p34$^{cdc2}$ rather than an indirect result of arresting cell cycle progression, the susceptibility of Jurkat cells to Fas ligation was analyzed after induction of $G_2$/M arrest with nocodazole (1 μg/ml). In contrast to cells expressing cdc2dn-HA or WEE1, nocodazole-arrested cells remained susceptible to Fas-mediated apoptosis (data not shown).

Genetic studies in the nematode *Caenorhabditis elegans* have identified two genes, *ced-3* and *ced-4*, that are required for programmed somatic cell deaths and a third gene, *ced-9*, that inhibits apoptosis induced by *ced-3* and *ced-4* (8, 17). The *ced-3* gene encodes a protein that shares sequence and functional similarity with the ICE family

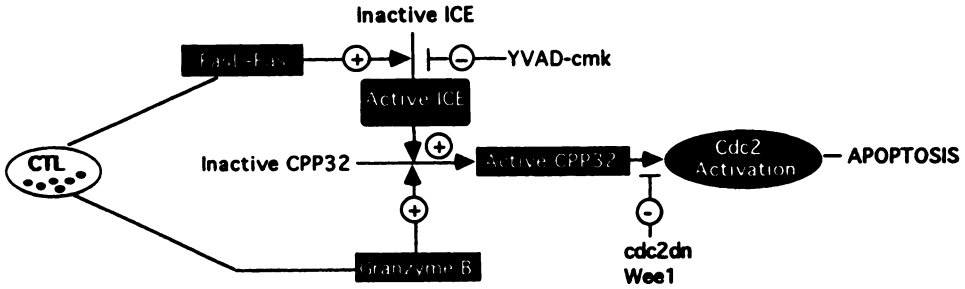


Fig. 4. Schematic representation of apoptosis mediated by CTLs. Induction of apoptosis by CTL involves granzyme-B-mediated activation of CPP32 (12) and p34$^{cdc2}$ (13). Ligation of the Fas/APO-1 receptor transduces an apoptotic signal that requires serial activation of ICE-like and CPP32-like proteases (4–6). The results of this study indicate that activation of p34$^{cdc2}$ by cysteine proteases may be a downstream effector of Fas/APO-1-induced apoptosis.

of cysteine proteases (ICE, CPP32$\beta$, and nedd-2/Ich-1 subfamilies; Refs. 8, 17). The proapoptotic effects of *ced-3* or ICE can be inhibited by expression of Bcl-2, the mammalian homologue of *ced-9*. Although apoptosis is regulated by diverse signals, these different signaling pathways converge at a cell death effector program that has been conserved in evolution. The *ced-3*/ICE-like cysteine proteases and *ced-9*/Bcl-2 proteins are believed to act at or close to the cell death effector level. The identification of the effector mechanisms responsible for induction of apoptosis by activated ICE/CPP32$\beta$-related proteases is under intense investigation. Recent studies have demonstrated that potentially important substrates of the ICE/CPP32$\beta$-like proteases include proteins involved in DNA repair [poly(ADP-ribose) polymerase and DNA-dependent protein kinase (DNA-PK$_{cs}$)] (18).

Our data indicate that Fas-mediated apoptosis requires ICE/CPP32$\beta$-mediated activation of p34$^{cdc2}$ kinase. Together with the demonstrated requirement of p34$^{cdc2}$ for granzyme-B-induced CPP32$\beta$-mediated apoptosis, our results suggest that activation of p34$^{cdc2}$ is a downstream determinant of apoptosis induced by ICE-related proteases (Fig. 4). The requirement for p34$^{cdc2}$ activation for Fas, as well as granzyme-B-induced apoptosis, provides a unified mechanism underlying CTL-mediated cytotoxicity that may explain the observed protection of target cells in the G0/G1 phase of the cell cycle against CTLs (19). Coupled with the similarity of the apoptotic phenotype with that of "mitotic catastrophes" resulting from unscheduled p34$^{cdc2}$ activation, our data reinforce the concept that the universal determinant of entry into mitosis may also be fundamentally intertwined with induction of apoptosis. This notion is consistent with the observations that activation of p34$^{cdc2}$ accompanies apoptosis induced by diverse signals, including CD3 ligation, tumor necrosis factor-$\alpha$, HIV-1 Tat protein, and DNA damage (20–23). Our studies indicate that p34$^{cdc2}$ activity is also an important determinant of p53-dependent DNA damage-induced apoptosis (24). We have demonstrated that inactivation of p34$^{cdc2}$ inhibits p53-dependent apoptosis after ionizing radiation (25). Although these studies suggest that the activity of p34$^{cdc2}$ is a critical modulator of apoptosis triggered by several stimuli, other reports have indicated that it may not be obligatory for all forms of cell death (26).

Because Fas-mediated apoptosis occurs in the presence of inhibitors of RNA or protein synthesis (4), the activation of p34$^{cdc2}$ by an ICE-like protease is probably a posttranscriptional event. This is consistent with the absence of any change in p34$^{cdc2}$ or cyclin B protein expression by Fas ligation. Because p34$^{cdc2}$ is not a substrate for ICE/CPP32$\beta$-related proteolysis, the induction of apoptosis by ICE-related proteases may involve cleavage of a cell cycle regulatory protein required to restrain the activity of p34$^{cdc2}$ kinase. Additional studies are required to identify the intermediate substrate(s) responsible for the regulation of p34$^{cdc2}$ by ICE-related proteases. Regardless of the specific mechanism involved, our data suggest that activation of p34$^{cdc2}$ is a fundamental component of the apoptosis effector mechanisms triggered by ICE-related proteases. In addition to providing a link between the biochemical and cell cycle regulatory determinants of apoptosis, the elucidation of the mechanism of Fas-mediated apoptosis should contribute to a better understanding of peripheral clonal deletion of lymphocytes or T-cell-mediated cytotoxicity, as well as provide insights for the treatment of diseases resulting from malfunction of the Fas system (1, 27).

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