Rescue from Granzyme B–induced Apoptosis by Weel Kinase

By Gao Chen, Lianfa Shi, David W. Litchfield, and Arnold H. Greenberg

Summary

Granzymes are a family of granule-associated serine esterases that mediate apoptosis by cytotoxic T lymphocytes and natural killer cells. We have previously shown that cdc2, the mitosis-regulating cyclin-dependent kinase, is required for granzyme B–induced apoptosis in target cells. In addition, granzyme B induces premature activation and tyrosine dephosphorylation of cdc2 during apoptosis. Throughout most of the cell cycle and until the cell is prepared to enter mitosis, cdc2 kinase activity is negatively regulated by phosphorylation of a residue within its adenosine triphosphate–binding domain by Weel, a nuclear kinase that maintains mitotic timing in eukaryotic cells.

We have transiently expressed c-myc epitope–tagged Weel cDNA in BHK cells. Cells that expressed Weel in the nucleus became resistant to apoptosis induced by granzyme B and perforin. Weel–transfected cells also exhibited markedly increased cdc2 tyrosine phosphorylation. Thus, Weel can rescue cells from granzyme-induced apoptosis by preventing cdc2 dephosphorylation.

Materials and Methods

Cells and Antibodies. Baby hamster kidney (BHK) cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 and 10% FCS. Anti-c-myc antibody was purchased from Santa Cruz Biotechnology Inc., (9E10; Santa Cruz, CA). Antiphosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (4G10, Lake Placid, NY). The rabbit anti-cdc2 antibody was produced by immunization with a COOH-terminal synthetic peptide as described previously (7).

Transient Transfections. Plasmid pWEE1 bearing a 5' c-myc epitope tag was obtained from Dr. Frank McKeon (Harvard University, Boston, MA) and has been described previously (12). BHK cells (5 × 10⁵) were plated onto 100 × 20-mm tissue culture dishes (Nunc, Roskilde, Denmark) in 10 ml of medium on day 1. On day 2, cesium chloride gradient–purified plasmid pWeel DNA was added to 500 μl of 0.25-M CaCl₂ and 20 mM Hepes and precipitated by dropping it slowly into 500 μl of 2x Hepes-buffered saline (2x Hepes-buffered saline is 282 mM NaCl, 0.78 mM Na₂HPO₄, and 50 mM Hepes, pH 7.1). After 20 min, 5 ml of complete medium was added to the mixture. The medium was aspirated from each dish, replaced with the DNA mixture, and allowed to incubate in a tissue culture incubator for 5 h. The cells were then washed twice with complete medium and incubated for an additional 12 h. BHK cells were then trypsinized, washed, and resuspended in HBSS containing 2 mM CaCl₂ and BSA (4 mg/ml, pH 7.4). Aliquots of 5 × 10⁵ cells in 80 μl were added to 80 μl of TEB (20 mM Tris-HCl, pH 7.2, 0.1 mM EGTA, and 140 mM...
NaCl) in 96-well V-bottomed microtiter plate containing granzyme B and perforin. After 2 or 3 h incubation at 37°C, cells were evaluated for c-myc/Weel staining and apoptosis.

**Immunofluorescence for c-myc/Weel Expression and Chromatin Condensation.** BHK cells were centrifuged in 96-well V-bottomed microtiter plates (5 × 10^5 cells per well); the medium was aspirated; and cells were fixed in 150 μl of 3% formaldehyde (Mallinckrodt Specialty Chemicals, Chesterfield, MO) in PBS and incubated for 10 min at room temperature. Cells were washed twice with PBS containing 0.1% NP-40 (Sigma Chemical Co., St. Louis, MO) (PBS–NP-40). Primary 9E10 antibody was diluted in PBS–NP-40 and incubated for 1 h while shaking, followed by two washes with PBS–NP-40. FITC-conjugated goat anti-mouse IgG secondary antibody (Sigma Chemical Co.) was also diluted in PBS–NP-40 and incubated for 30 min. DNA was labeled using Hoechst dye at 1 μg/ml in PBS–NP-40 for 1 h. Cells were resuspended in 10 μl of 90% glycerol, 10% 0.2 M Tris (pH 8.0), mounted on glass slide, and examined on an axiovert fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Between 150 and 300 cells were counted for each incubation condition.

**Western Blotting and Immunoprecipitation.** 10^6 BHK cells treated with granzyme B and perforin as described above were washed twice with cold PBS and extracted on ice with 1 ml of extraction buffer (50 mM β-glycerophosphate, pH 7.3, 1% NP-40, 10 mM NaF, 1% aprotinin, and 1 mM sodium vanadate) for 1 h. The lysates were centrifuged at 10,000 g for 20 min at 4°C, and the cdc2 was immunoprecipitated with 3 μl of a rabbit antibody raised against the COOH-terminal peptide of cdc2 and 30 μl of protein A beads (Bio-Rad Laboratories, Richmond, CA) and rotated at 4°C for 1 h. In some experiments, excess cdc2 peptide was added to block precipitation as described previously (7). Precipitates were washed three times in buffer, and samples were fractionated on an SDS-PAGE 12% mini-gel (Bio-Rad Laboratories) and electrophoretically transferred to nitrocellulose membrane (BA-5 NC; Schleicher & Schuell, Inc., Keene, NH) using a multiphore II electrophoresis system (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The membranes were blocked for 1 h in PBS containing 2% BSA and incubated in antiphosphotyrosine 4G10 antibody diluted into the same solution for 1 h. After three washes in blocking solution, membranes were incubated with goat anti-mouse conjugated to horseradish peroxidase for 1 h. Membranes were then washed four times and developed using chemiluminescence substrates according to manufacturer's instructions (ECL; Amersham Corp., Arlington Heights, IL). For c-myc/Weel staining, 25 μl of cell lysate was fractionated on SDS-PAGE and transferred to nitrocellulose as above. Membranes were incubated with 9E10 mAb and developed as above.

**Discussion**

In these experiments, we have shown that wee1 overexpression protects cells from granzyme B-induced apoptosis. The human p50Weel exhibits homology to both wee1 and mik1 (14), which are redundant kinases in Schizosaccharomyces pombe (15), and has been shown to rescue BHK cells overproducing the phosphatase cdc25, or cdc2 and cyclin A or B (12). The coincident suppression of apoptosis and cdc2 phosphorylation by Weel, combined with our earlier findings that suppression of cdc2 expression abrogates apoptosis (7), indicates that cdc2 activation and dephosphorylation are necessary for direct fluorescence microscopy, and this was localized to the nucleus (Fig. 1, A and C). Weel1 or vector-transfected cells were treated with granzyme B and perforin. After 2 or 3 h incubation, a high proportion of the vector-transfected cells and Weel-transfected cells that did not express Weel1 were apoptotic with intense chromatin condensation detected by Hoechst staining (Fig. 2). However, in Weel-expressing cells, we found only low levels of chromatin condensation just above the background of non-granzyme-treated cells, indicating that granzyme B-induced apoptosis had been almost completely inhibited (Fig. 1, A and C; Fig. 2). It was also found that cells that were calcium phosphate transfected with the empty vector exhibited levels of apoptosis between 20 and 22%. Simple calcium phosphate treatment without the vector produced a similar result. This apoptosis was also reduced by up to 50% after Weel transfection, and the total viable cell recovery was increased, indicating that Weel was protecting cells from apoptosis induced during calcium phosphate-mediated transfection (Fig. 2).

**Weel Phosphorylates cdc2 in Granzyme B–Treated BHK Cells.** To determine the effect of Weel on cdc2 phosphorylation, we transfected BHK cells with either Weel or vector, and then incubated with granzyme B and perforin as before. Cell lysates were precipitated with an antibody to the COOH terminus of cdc2, and the precipitate was examined by SDS-PAGE and Western blotting with either anti-cdc2 antibody or antiphosphotyrosine antibody 4G10. Western blots of cdc2 tyrosine phosphorylation showed a dramatic increase in the Weel-transfected cells (Fig. 3, upper panel). The identity of the cdc2 in the precipitates was confirmed using peptide inhibition of immunoprecipitation (Fig. 3, lane 4). Weel1 and vector-transfected controls were examined by Western blotting for c-myc, and the predicted 50-kD band was detected only in the Weel-transfected cells (Fig. 3, lower panel).

Because we had detected apoptosis during vector transfection by calcium phosphate treatment of BHK cells and rescue with Weel, we examined the state of cdc2 phosphorylation in these cells. It was evident that cdc2 tyrosine phosphorylation decreased significantly after calcium phosphate transfection, and that Weel transfection restored this to equal or slightly greater levels than those seen in untreated BHK cells (Fig. 4, upper panel). The level of cdc2 protein was unchanged in cells transfected with Weel, indicating that the variations in cdc2 phosphorylation were not due to changes in cdc2 protein levels (Fig. 4, lower panel).
Figure 1. BHK cells expressing c-myc epitope-tagged wee1 are resistant to granzyme B and perforin. (A, C) Hoechst dye staining (B, D) anti-myc antibody. The Wee1 is detected by anti-myc antibody and is expressed in the nucleus of transfected cells. After addition of granzyme B (1,500 ng/ml) and perforin (65 ng/ml), BHK cells not expressing Wee1 undergo apoptosis identified by chromatin condensation using Hoechst dye (solid arrow). Cells expressing Wee1 in the nucleus have a normal chromatin structure (open arrow). (A, B) ×400; (C, D) ×1,000.

Figure 2. Quantitation of apoptosis in BHK cells expressing wee1. Control transfected BHK cells induced to undergo apoptosis by granzyme B and perforin at different doses and times of treatment (Control). Cells that were transfected with and expressed c-myc epitope–tagged Wee1 are resistant to apoptosis induced by granzyme B (Wee1+), while those cells not expressing wee1 in the same transfection were susceptible (Wee1−). In cells not treated with granzyme B, calcium phosphate transfection induced low levels of apoptosis, which were suppressed by Wee1 transfection. Granzyme B was added at the doses indicated, and the perforin concentration was constant at 65 ng/ml. Between 150 and 300 cells were counted for each condition. The experiment was repeated several times with similar results.
Figure 3. Weel maintains phosphorylation of cdc2 during granzyme B-induced apoptosis (upper panel). Phosphorylation of cdc2 by weel in granzyme B-treated BHK cells. Cells were transfected with Weel (lanes 2-4) or control vector (lane 1), and then treated with granzyme B and perforin (lanes 1-4). Cell lysates were precipitated with anti-cdc2 antibody, and membranes were blotted with antiphosphotyrosine mAb. In lane 4, excess cdc2 COOH-terminal peptide was added to inhibit cdc2 precipitation. myc-epitope tagged Weel expression (lower panel). Lysates of vector-transfected (lane 1) and Weel-transfected BHK cells (lanes 2-4) were blotted with anti-c-myc mAb.

It has been suggested that an increase in cytoplasmic Ca$^{2+}$ is an early signal for initiation of apoptosis by a number of agents (19), although it is often not sufficient by itself (20). Thus, it is possible that Ca$^{2+}$ may contribute to other apoptosis signals through effects on cdc2 phosphorylation. Granzyme B and perforin also require Ca$^{2+}$ to induce apoptosis (21).

Although these data support the hypothesis that weel-induced cdc2 phosphorylation can rescue cells from apoptosis, it cannot be concluded that this is the only effect of weel. Premature induction of cyclin A-associated cdk2 activity has also been observed in apoptosis (22), and p58 PITSLRE, a distantly related cdc2 gene family member, induces apoptosis when overexpressed (23). Therefore, we cannot rule out the possibility that other cyclin-dependent kinases (CDKs) participate in granzyme B-induced apoptosis, and that Weel overexpression is phosphorylating and inhibiting these kinases in addition to cdc2. In either case, weel appears to play a key role in both control of mitotic timing and apoptosis mediated by CDKs.

Recent evidence indicates that there must be more than one effector pathway for mediating apoptosis, as cdc2 is used in some but not all forms of apoptosis. Programmed cell death in postmitotic neurons can occur even though cdc2 is not expressed (24), and thymocytes treated with etoposide or dexamethasone undergo apoptosis but do not contain increased levels of cdc2 kinase activity (25). On the other hand, in addition to our reports that premature cdc2 activation is required for granzyme-induced apoptosis, Meikrantz et al. (22) found increased cdc2 and cdk2 kinase activity associated with cyclin A after TNF-α and drug-induced apoptosis in hydroxyurea-arrested HeLa cells. Similarly, in cells undergoing apoptosis after treatment with taxol, cdc2 kinase activity is coincidently increased (26). Recently, using the model of activation-induced apoptosis after T cell receptor cross-linking of a T cell hybridoma, Fotedar et al. (27) have shown that cdc2/cyclin B activity is increased, and, using antisense oligonucleotides, that cyclin B expression is necessary for apoptosis. Interestingly, they were unable to block dexamethasone-induced apoptosis, indicating that, in the same cell, only one form of apoptosis requires cdc2/cyclin B. Considering all of these data, it is likely that several members of the CDK family are effectors of apoptosis, including cdc2, cdk2, and p58PITSLRE, although it is not known if they act independently of one another.

If there are distinct CDK effector molecules for different forms of apoptosis, then the view that there is a necessary and sufficient biochemical event for all forms of apoptosis may be challenged. Strasser et al. (28) have recently identified p53-independent apoptosis in T cells undergoing DNA damage, which again supports the idea that there is more than one apoptosis pathway for DNA damage. The family of three IL-1β-converting enzyme (ICE) proteases, ICE (29), ICH-1 (30), and CPP32 (31), that are the mammalian counterparts of ced-3 of Caenorhabditis elegans may constitute a
common apoptosis pathway. However, with several members of the ICE family and several members of the CDK family implicated in apoptosis, it is equally plausible that more than one pathway exists and that individual members of these two families are used for initiating apoptosis in different cells or following specific initiating signals. It is also not yet clear whether or not the CDK and ICE families are acting on the same pathways. In conclusion, our results indicate that one effector pathway, which is used by the granzymes, initiates apoptosis by the targeted disruption of cdc2 phosphorylation.

We thank Frank McKeon for the gift of the myc-tagged Weel cDNA and Deli He for her technical assistance.

A H Greenberg is a Terry Fox Scientist of the National Cancer Institute of Canada. D W Litchfield is a Scholar of the National Cancer Institute of Canada. This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada.

Address correspondence to Dr. Arnold H. Greenberg, Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg, MB, R3E OV9, Canada.

Received for publication 4 January 1995 and in revised form 21 February 1995.

References
1. Shi, L., C.M. Kam, J.C. Powers, R. Aebersold, and A.H. Greenberg. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. J. Exp. Med. 176:1521-1529.
2. Shiver, J.W., L. Su, and P.A. Henkart. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolytin and granzyme A. Cell. 71:315-322.
3. Heusel, J.W., R.L. Wesselschmidt, S. Shresta, J.H. Russell, and T.J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell. 76:977-987.
4. Kaege, D., B. Lederman, K. Buerki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature (Lond.). 369:31-37.
5. Lawin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature (Lond.). 370:650-652.
6. Walsh, C.M., M. Marlozina, C.C. Liu, R. Ueda, C.G. Kurahara, J.L. Christensen, M.T.F. Huang, J.D.E. Young, R. Ahmed, and W.R. Clark. 1994. Immune function in mice lacking the perforin gene. Proc. Natl. Acad. Sci. USA. 91:10854-10858.
7. Shi, L., W.K. Nishioka, J. Th'ng, E.M. Bradbury, D.W. Litchfield, and A.H. Greenberg. 1994. Premature p34 CDC2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34CDC2 kinase activation in vertebrates. EMBO (Eur. Mol. Biol. Organ.) J. 10:3331-3341.
8. Nurse, P. 1990. Universal control mechanism regulating onset of cell division. Nature (Lond.). 344:503-508.
9. Parker, L.L., S.A. Walter, P.G. Young, and H. Piwnica-Worms. 1991. Negative regulator Wee1 by the nim1/cdrl kinase. Cell. 76:919-929.
10. Coleman, T.R., Z. Tang, and W.G. Dunphy. 1993. Negative regulation of the weel protein kinase by direct action of the nim1/cdrl mitotic inducer. Cell. 72:919-929.
11. Duke, R.C., R.Z. Witter, P.B. Nash, J.D.E. Young, and D.M. Ojcius. 1994. Cytolysis mediated by ionophores and pore-forming agents: role of intracellular calcium in apoptosis. FASEB (Fed. Am. Soc. Exp. Biol.) J. 3:1843-1849.
12. Heald, R., M. McLoughlin, and F. McKeon. 1993. Human Weel maintains mitotic timing by protecting the nucleus from cytologically activated Cdc2 kinase. Cell. 74:463-474.
13. Krek, W., and E.A. Nigg. 1991. Mutations of p34CDC2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34CDC2 kinase activation in vertebrates. EMBO (Eur. Mol. Biol. Organ.) J. 10:3331-3341.
14. Igarashi, M., A. Nagata, S. Jinno, K. Suto, and H. Okoyama. 1994. Weel(+)like gene in human cells. Nature (Lond.). 353:80-83.
15. Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirshner, and D. Beach. 1991. micl and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell. 64:1111-1122.
16. Wu, L., and P. Russell. 1993. Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. Nature (Lond.). 363:738-741.
17. Parker, L.L., S.A. Walter, P.G. Young, and H. Piwnica-Worms. 1993. Phosphorylation and inactivation of the mitotic inhibitor Weel by the nim1/cdrl kinase. Nature (Lond.). 363:736-738.
18. Coleman, T.R., Z. Tang, and W.G. Dunphy. 1993. Negative regulation of the weel protein kinase by direct action of the nim1/cdrl mitotic inducer. Cell. 72:919-929.
19. Meconkey, D.J., P. Hartzell, P. Nicotera, and S. Orrenius. 1989. Calcium-activated DNA fragmentation kills immature thymocytes. FASEB (Fed. Am. Soc. Exp. Biol.) J. 3:1843-1849.
20. Duke, R.C., R.Z. Witter, P.B. Nash, J.D.E. Young, and D.M. Ojcius. 1994. Cytolysis mediated by ionophores and pore-forming agents: role of intracellular calcium in apoptosis. FASEB (Fed. Am. Soc. Exp. Biol.) J. 8:237-246.
21. Shi, L., R.P. Kraut, R. Aebersold, and A.H. Greenberg. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J. Exp. Med. 175:553-566.
22. Meikrantz, W., S. Gisselbrecht, S.W. Tam, and R. Schlegel. 1994. Activation of cyclin A-dependent protein kinases during apoptosis. Proc. Natl. Acad. Sci. USA. 91:3754-3758.
1994. Activation of p34cdc2 coincident with taxol-induced apoptosis. *Cell Growth & Differ.* 5:1041-1050.

Freeman, R.S., S. Estus, and E.M. Johnson, Jr. 1994. Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of cyclin D1 during programmed cell death. *Neuron.* 12:343-355.

Norbury, C., M. MacFarlane, H. Fearnhead, and G.M. Cohen. 1994. CDC2 activation is not required for thymocyte apoptosis. *Biochem. Biophys. Res. Commun.* 202:1400-1406.

Lahti, J.M., J. Xiang, L.S. Heath, D. Campana, and V.J. Kidd. 1995. PITSLRE protein kinase activity is associated with apoptosis. *Mol. Cell. Biol.* 15:1-11.

Fotedar, R., J. Flatt, S. Gupta, R.L. Margolis, P. Fitzgerald, H. Messier, and A. Fotedar. 1994. Activation induced T cell death is cell cycle dependent and regulated by cyclin B. *Mol. Cell. Biol.* 15:932-942.

Strasser, A., A.W. Harris, T. Jacks, and S. Cory. 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell.* 79:329-339.

Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 Beta-converting enzyme. *Cell.* 75:641-652.

Wang, L., M. Miura, L. Bergeron, H. Zhu, and J. Yuan. 1994. *Ich-1*, and *Ice/ced-3*-related gene, encodes both positive and negative regulators of programmed cell death. *Cell.* 78:739-750.

Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 Beta-converting enzyme. *J. Biol. Chem.* 269:30761-30764.