0.05% sodium azide], and analyzed for constant time (120 s) on a FACScan cytometer with Lysis II software (Becton Dickinson, Mountain View, CA). For cell cycle analysis, we used 2×10^6 A.E7 cells stimulated for 66 hours by peptide antigen and 2×10^7 splenic APCs. For the last 18 hours BrdU (1 μM) was added. Viable cells were separated by Ficoll gradients, ethanol-fixed, and stained with mAbs to BrdU conjugated to FITC according to manufacturer's instructions (Becton Dickinson). We collected 10,000 events and the data is representative of four experiments.

18. J. Critchfield, M. Racke, M. J. Lenardo, unpublished results. Death was not due to T-T cytotoxicity because it was observed at a high APC to T cell ratio, and mouse T cells lack major histocompatibility complex class II expression. Also, neither the antigen solution itself nor supernatants taken from cultures stimulated with high antigen concentrations were intrinsically toxic.
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20. The MBP-reactive LNTCs were stimulated in vitro with 50 μM Ac1–11 for 48 hours, then purified on lympholyte M (Cedarlane Laboratories, Westbury, NY) gradients. Activated cells were incubated with the indicated amounts of IL-2 for 48 hours. At this time, [^{3}H]thymidine incorporation was measured. The cells were then rinsed and restimulated with 1×10^4 LNTCs with 50-fold excess of irradiated syngeneic (B10.PL) splenocytes per U-bottom well and the indicated concentrations of Ac1–11 peptide. During restimulation IL-2 was included to prevent IL-2–withdrawal apoptosis. Cells were quantitated after 48 hours. Control experiments showed that the Ac1–11 peptide had no intrinsic toxicity on LNTCs. For experiments that used mAbs 3C7 or S4B6, 5×10^4 A.E7 cells were incubated with 5×10^5 splenic APCs, and the indicated amount of peptide antigen and mAb (50 μg per well) was added in a 96-well flat bottom dish. We used A.E7 cells because they enter a resting state and do not undergo withdrawal apoptosis when IL-2 is removed from the culture. Viable cell numbers are the means from duplicate values quantitated by flow cytometry (*10*). The mAb 3C7 [T. R. Malek, G. Ortega, J. P. Jakway, C. Chan, E. M. Shevach, *J. Immunol.* **133**, 1976 (1984)] or S4B6 [T. R. Mosmann, H. Cherwinski, M. W. Bond, M. A. Giedlin, R. L. Coffman, *ibid.* **136**, 2348 (1986)] was purified from ascites by ammonium sulfate precipitation and dialyzed against PBS. The mAb 3C7 was then purified by Sephadex gel filtration chromatography and filtered for sterility. The mAb 11B11 was described previously [J. O'Hara and W. E. Paul, *Nature* **315**, 333 (1985)].
21. The MBP-reactive TCR transgenic splenocytes from unprimed animals were stimulated in vitro with 50 μM Ac1–11 peptide for 96 hours and rinsed, and 3×10^7 cells in 200 μl of PBS were injected into B10.PL or PL/J hosts. Ten days after transfer, cell suspensions from mesenteric and aortic lymph nodes or spleen were stained with FITC-conjugated mAb to V_β8.1,2, PE-conjugated mAb to V_α2 (Pharmingen), and Red 613–conjugated mAb to CD4 (Gibco-BRL), then 5×10^4 $CD4^+$ events were analyzed by FACScan with Lysis II software (Becton Dickinson). Transgenic animals were used from the second or third backcross generation; no evidence of rejection or graft versus host disease was observed. Experiments were done under an approved protocol in accordance with the animal use guidelines of the National Institutes of Health.
22. Nontransgenic animals have been shown to have a limited but heterogeneous group of MBP-reactive TCRs. [J. L. Urban *et al.*, *Cell* **54**, 577 (1988); R. B. Bell, J. W. Lindsey, R. A. Sobel, S. Hodgkinson, L. Steinman, *J. Immunol.* **150**, 4085 (1993)].
23. Donor mice, $(PL \times SJL)F_1$, 8- to 12-week-old females (Jackson Laboratory, Bar Harbor, ME) were primed at the shoulders and flanks with 400 μg of guinea pig MBP in complete Freund's adjuvant as described [M. K. Racke *et al.*, *J. Immunol.* **146**, 3012 (1991)]. Ten days later, draining LNCs were harvested and 8×10^6 cells in 2 ml were stimulated with MBP (25 μg/ml) for 4 days. Cells (3×10^7) in PBS (200 μl) were immediately injected intravenously into syngeneic, naive recipients. Statistical analysis was done by two-sample *t* tests and a Hotelling's T^2 multivariate analysis. Histopathological analysis was done at day 60 after transfer on the dorsal spinal cord at L7 from mice perfused through the heart with 2.5% glutaraldehyde. The histological samples were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Photomicrographs (magnification ×271) were taken from 1-μm sections stained with toluidine blue. Encephalitogenic nontransgenic LNTCs were prepared, then rinsed in RPMI 1640 with 50 mM Hepes (pH 7.5) and stained in 10 ml of DiI (4 μg/ml) (Molecular Probes, Inc., Eugene, OR) for 10 min at 37°C. Cells were then washed three times with RPMI 1640 with 10% fetal calf serum before transfer. Cells were counterstained with FITC-conjugated antibody to CD4 (Pharmingen) and flow cytometry was done (*21*).
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Premature $p34^{cdc2}$ Activation Required for Apoptosis

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Activation of the serine-threonine kinase $p34^{cdc2}$ at an inappropriate time during the cell cycle leads to cell death that resembles apoptosis. Premature activation of $p34^{cdc2}$ was shown to be required for apoptosis induced by a lymphocyte granule protease. The kinase was rapidly activated and tyrosine dephosphorylated at the initiation of apoptosis. DNA fragmentation and nuclear collapse could be prevented by blocking $p34^{cdc2}$ activity with excess peptide substrate, or by inactivating $p34^{cdc2}$ in a temperature-sensitive mutant. Premature $p34^{cdc2}$ activation may be a general mechanism by which cells induced to undergo apoptosis initiate the disruption of the nucleus.

On contact with target cells, cytotoxic T lymphocytes (CTLs) release granule serine proteases that trigger apoptosis (*1–3*). The transmembrane pore-forming protein perforin probably facilitates protease entry into the target cell (*2, 3*). Unlike developmentally regulated programmed cell death, apoptosis induced by cytotoxic granule proteases does not require new protein synthesis (*2, 3*), suggesting that the protease initiates nuclear disintegration through a posttranslational mechanism. The $p34^{cdc2}$ kinase is a highly regulated serine-threonine kinase (*4*) that, when complexed with cyclins A and B, controls cell entry into mitosis; this complex initiates the dissolution of the nuclear membrane and promotes chromatin condensation, events that are also hallmarks of apoptosis (*5, 6*). The resemblance of apoptosis to the "mitotic catastrophe" seen in eukaryotic cells overexpressing $p34^{cdc2}$ at an inappropriate time during the cell cycle (*7, 8*) prompted us to examine the role of this kinase in apoptosis.

We examined the induction of $p34^{cdc2}$ kinase in YAC-1 lymphoma cells by fragmentin-2 in the presence or absence of perforin. Fragmentin-2 is a granule serine protease produced by natural killer (NK) cells that has homology to human cytotoxic T lymphocyte (CTL) granzyme B (*2*). After a 45-min treatment with these agents, $p34^{cdc2}$ was immunoprecipitated from the cell lysates with a polyclonal COOH-terminal specific antibody (*9*) and the kinase activity was measured with two $p34^{cdc2}$ peptide substrates [peptide A, derived from casein kinase II (CKII) (*9*), and peptide B, derived from nucleolin (*10*)] (*11*). In the presence of constant amounts of perforin, fragmentin-2 stimulated kinase activity in a

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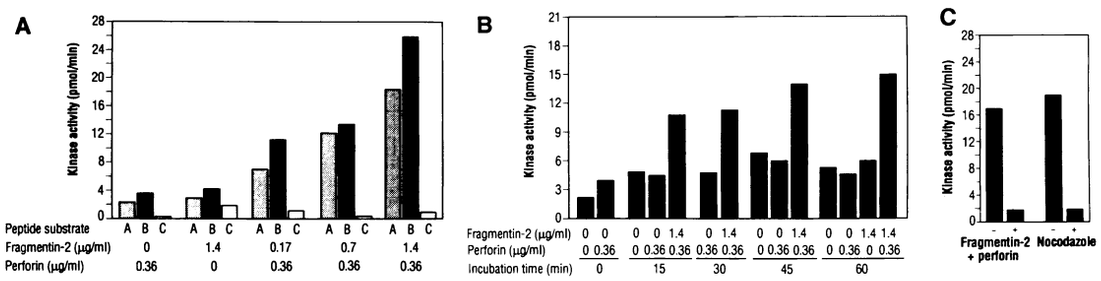

Fig. 1. (**A**) Activation of $p34^{cdc2}$ kinase in YAC-1 cells induced to undergo apoptosis by the fragmentin-2 protease. Cells were treated with fragmentin-2 and perforin for 45 min and then the lysates were immunoprecipitated with an antibody to $p34^{cdc2}$ and assayed for kinase activity (*11*) with three substrates: peptide A (Gln-Leu-Gln-Leu-Gln-Ala-Ala-Ser-Asn-Phe-Lys-Ser*-Pro-Val-Lys-Thr-Ile-Arg; gray bars); peptide B (Ala-Val-Thr*-Pro-Ala-Lys-Lys-Ala-Ala-Thr*-Pro-Ala-Lys-Lys-Ala; black bars), which are known $p34^{cdc2}$ substrates (*9, 10*); and peptide C (a control peptide in which the prolines in peptide B at positions 4 and 11 had been replaced with glycines; open bars). (**B**) Time-dependent activation of $p34^{cdc2}$ kinase by fragmentin-2 and perforin. Cells were treated for the indicated length of time and kinase activity assayed with peptide A as in Fig. 1A. (**C**) Comparison of $p34^{cdc2}$ kinase activity after fragmentin-2 and nocodazole treatment. YAC-1 cells were treated with fragmentin-2 (1.4 μg ml^{-1}) and perforin (0.36 μg ml^{-1}) for 45 min, or nocodazole (0.05 μg ml^{-1}) for 8 hours and then immunoprecipitated with antibody to $p34^{cdc2}$ in the presence (+) or absence (−) of the COOH-terminal peptide (50 μg ml^{-1}) used to raise the $p34^{cdc2}$-specific antibody. Kinase activity was measured with peptide A.

dose-dependent manner. A control substrate with glycines at positions 4 and 11 of peptide B (peptide C), which changes the $p34^{cdc2}$ recognition sequence, was not phosphorylated either by purified activated $p34^{cdc2}$ (*12*) or by $p34^{cdc2}$ immunoprecipitated from cells undergoing apoptosis induced by fragmentin-2 (Fig. 1A). The induction of kinase activity by fragmentin-2 and perforin was extremely rapid. It was detected by 15 min and reached maximal levels after 60 min (Fig. 1B), coinciding with the rate of appearance of DNA fragmentation and apoptosis induced by fragmentin-2 (*2*). Kinase activity at 60 min was equivalent to that in cells arrested in G_2/M by nocodazole for 8 hours (Fig. 1C). If peptide corresponding to the COOH-terminal epitope used to raise the antibody to $p34^{cdc2}$ was included in the immunoprecipitates, no kinase activity was recovered (Fig. 1C). The proteolytic activity of the fragmentin-2 was required for $p34^{cdc2}$ activation, as the relevant protease inhibitor Boc-Ala-Ala-Asp-CH_2Cl, but not the control inhibitor D-Phe-Pro-Arg-CH_2Cl, blocked activation (*13*).

The $p34^{cdc2}$ kinase activity is regulated by its association with cyclins and by phosphorylation on Ser, Thr, and Tyr residues (*14*). When phosphorylated on Tyr^{15}, $p34^{cdc2}$ is inactive until dephosphorylation results in its activation at the G_2/M transition (*14*). To ascertain the Tyr phosphorylation state of $p34^{cdc2}$ in YAC-1 cells treated with fragmentin-2 and perforin, we immunoprecipitated the kinase and examined it by immunoblotting with an antibody to phosphotyrosine. The $p34^{cdc2}$ kinase was dephosphorylated in cells treated with fragmentin-2 and perforin for 45 min to the same extent as it was in cells arrested at G_2/M for 8 hours by nocodazole (Fig. 2). The reduction in $p34^{cdc2}$ phosphorylation was not due to proteolytic degradation of the kinase, as parallel extracts contained similar amounts of kinase protein (Fig. 2).

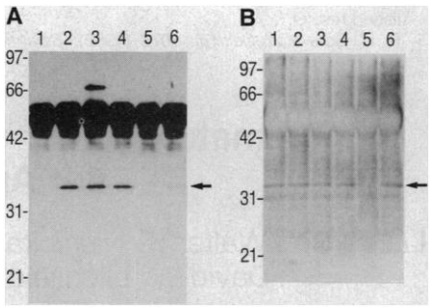

Fig. 2. Tyrosine dephosphorylation of $p34^{cdc2}$ during kinase activation by fragmentin-2 protease. (**A**) Immunoblots developed with an antibody to phosphotyrosine (4G10) (*25*) or (**B**) a rabbit antibody to $p34^{cdc2}$ (*14*). YAC-1 cells were incubated under the following conditions: Lane 1, fragmentin-2 (1.4 μg ml^{-1}) and perforin (0.36 μg ml^{-1}); lane 2, fragmentin-2 only; lane 3, perforin only; lane 4, YAC-1 untreated control; lane 5, YAC-1 control with excess COOH-terminal $p34^{cdc2}$ peptide added to lysates before immunoprecipitation; lane 6, nocodazole (0.05 μg ml^{-1}). Samples in lanes 1 to 6 were incubated at 37°C for 45 min; the sample in lane 6 was incubated for 8 hours. Molecular size markers are indicated on the left (in kilodaltons).

To determine whether the induction of $p34^{cdc2}$ kinase activity was required for DNA damage, we preincubated fragmentin-2 and perforin with excess peptide A to compete for $p34^{cdc2}$ kinase phosphorylation of critical substrates. DNA fragmentation was inhibited at 50% of maximum [median inhibitory dose (ID_{50}) = 50 μM] (Fig. 3). Several other CKII-derived peptides that are less efficient $p34^{cdc2}$ substrates than peptide A were not inhibitory. None of the peptides had any effect on perforin-mediated membrane damage (Fig. 3) or fragmentin-2 protease activity (*15*), indicating that the inhibition of apoptosis was not due to inactivation of either of these proteins.

In order to establish more conclusively that fragmentin-2–induced apoptosis required $p34^{cdc2}$, we used the murine mammary carcinoma cell line FT210, which contains a temperature-sensitive mutation in $p34^{cdc2}$ (*16*). At restrictive temperatures, kinase activity was reduced by 75% as a result of $p34^{cdc2}$ degradation (*16, 17*), and the cells became resistant to fragmentin-2–induced apoptosis. Little DNA solubilization or oligonucleosomal DNA ladder formation could be detected (Fig. 4), and chromatin condensation was substantially reduced when assayed by Hoechst dye staining (*18*). By contrast, parental FM3A mammary carcinoma cells remained sensitive under all conditions (Fig. 4). Similar results were obtained with fragmentin-3 (*2, 19*). We excluded the possibility that G_2/M arrest rather than loss of $p34^{cdc2}$ resulted in resistance to fragmentin-2 and -3 by showing that nocodazole-arrested cells remained completely susceptible to apoptosis by these proteases (*12*).

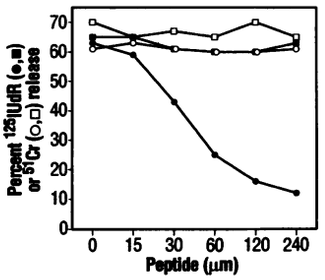

Fig. 3. Inhibition of apoptosis by excess $p34^{cdc2}$ peptide substrate. Peptide A (circles), which corresponds to the $p34^{cdc2}$ phosphorylation site on CKII (*9*), or another CKII-derived peptide that is a less efficient $p34^{cdc2}$ substrate (I) (squares) (Ile-Ser-Ser-Val-Pro-Tyr-Pro-Ser-Pro-Leu-Gly-Pro-Leu-Ala-Gly), was added to YAC-1 cells in the presence of fragmentin-2 and perforin. Apoptosis was measured by 125-iododeoxyuridine (^{125}IUdR) release (filled symbols) after 45 min. The effect of the peptides on perforin activity was also measured by membrane damage in a ^{51}Cr-release assay (*2*) (open symbols) after 45 min.

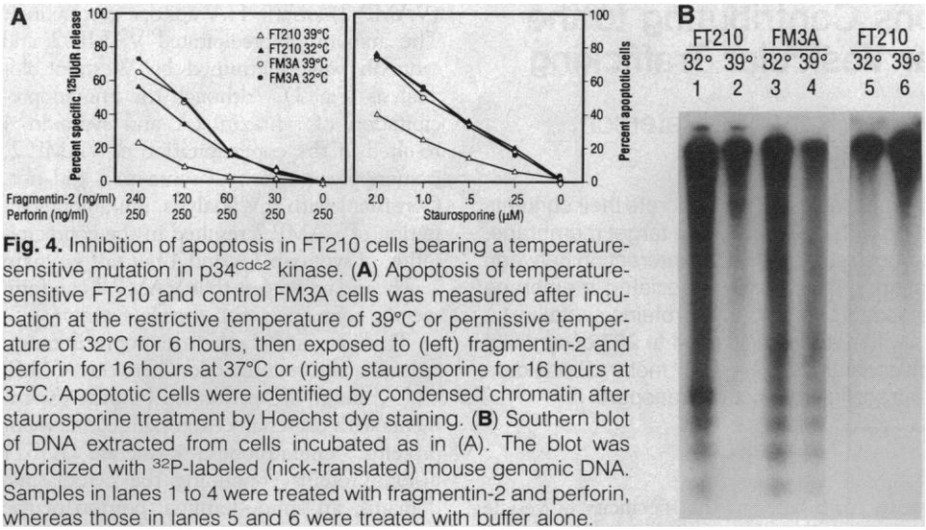

Fig. 4. Inhibition of apoptosis in FT210 cells bearing a temperature-sensitive mutation in p34^{cdc2} kinase. (**A**) Apoptosis of temperature-sensitive FT210 and control FM3A cells was measured after incubation at the restrictive temperature of 39°C or permissive temperature of 32°C for 6 hours, then exposed to (left) fragmentin-2 and perforin for 16 hours at 37°C or (right) staurosporine for 16 hours at 37°C. Apoptotic cells were identified by condensed chromatin after staurosporine treatment by Hoechst dye staining. (**B**) Southern blot of DNA extracted from cells incubated as in (A). The blot was hybridized with ^{32}P-labeled (nick-translated) mouse genomic DNA. Samples in lanes 1 to 4 were treated with fragmentin-2 and perforin, whereas those in lanes 5 and 6 were treated with buffer alone.

The idea that p34^{cdc2} may be an effector of other forms of apoptosis was examined in FT210 cells by treatment with the protein kinase inhibitor staurosporine, which activates an apoptosis pathway regulated by *bcl-2* (*20*). As measured by chromatin condensation, there were substantially fewer apoptotic FT210 cells at the restrictive temperature; FM3A cells, in contrast, were unaffected by the temperature shift (Fig. 4).

Among the characteristic morphological changes observed in apoptosis induced by CTLs and NK cells are chromatin condensation (*21*), and nuclear envelope breakdown which is associated with lamin phosphorylation and solubilization (*22*). During a normal cell cycle, but restricted to the G_2/M transition, p34^{cdc2} kinase activation initiates nuclear membrane dissolution by lamin phosphorylation and disassembly (*4*, *5*) and promotes chromosome condensation (*14*). We have identified a rapid activation of p34^{cdc2} kinase by the NK cell granule protease fragmentin-2 under conditions that induce apoptosis. The rapidity of activation contrasts with the time required to detect equivalent cyclin-dependent p34^{cdc2} activity at G_2/M in nocodazole-arrested cells, indicating that p34^{cdc2} kinase activity is being uncoupled from its normal cell cycle controls. The dissociation of p34^{cdc2} kinase activation from mitosis has been seen in cdc2-Y15F phosphorylation mutants and *wee1*/*mik1* kinase mutants in *Schizosaccharomyces pombe* (*7*), and in baby hamster kidney (BHK) cells overexpressing transfected p34^{cdc2} and cyclin A or B (*8*). The consequence of this dissociation has been appropriately referred to as a "mitotic catastrophe," and the morphological characteristics are markedly similar to the features of apoptosis (*7*, *8*).

The initiation of abortive mitosis through premature p34^{cdc2} activation may be a general mechanism for the induction of apoptosis. We have demonstrated that apoptosis induced by fragmentin-2 or by staurosporine requires p34^{cdc2}. We have also shown that when NIH 3T3 cells are in G_0, a stage at which p34^{cdc2} is not expressed, they are resistant to CTL-induced apoptosis (*23*). After c-*myc* or *p53* transfection into fibroblasts or tumor cells (*24*), the loss of cell cycle control may lead to the inappropriate activation of p34^{cdc2} with the consequent lethal effects on the nucleus. The idea that p34^{cdc2} activation could be a convergence point of different apoptotic signals is an appealing explanation for the observation that most forms of apoptosis terminate with nuclear dissolution.

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11. Samples containing 10^6 YAC-1 cells in 0.5 ml of Hanks' balanced salt solution (HBSS), 2 mM $CaCl_2$, and bovine serum albumin (BSA; 4 mg ml^{-1}) (pH 7.4) were added to 0.5 ml of fragmentin-2, perforin prepared in 140 mM NaCl, 10 mM Hepes, and 1 mM EGTA (pH 7.4), or to both. After incubation at 37°C, 0.25 ml of ice-cold 5× concentrated lysis buffer was added to a final concentration of 50 mM β-glycerophosphate (pH 7.3), 1% Nonidet P-40, 10 mM NaF, 1% aprotinin, and 1 mM sodium vanadate. The lysates were centrifuged at 30,000*g* for 10 min at 4°C and the p34^{cdc2} was immunoprecipitated with 5 μl of a rabbit antibody raised against the COOH-terminal peptide of p34^{cdc2} and 30 μl of protein A beads (Bio-Rad). Precipitates were washed three times in buffer and assayed for kinase activity as described in (*9*). Each assay contained 1×10^5 cell equivalents.
12. L. Shi *et al.*, unpublished data.
13. Fragmentin-2 (1.4 μg ml^{-1}) was incubated with or without 200 μM Boc-Ala-Ala-Asp-CH_2Cl or 200 μM D-Phe-Pro-Arg-CH_2Cl for 15 min at room temperature and then added to YAC-1 cells and perforin (0.36 μg ml^{-1}) for a further 60 min at 37°C. The p34^{cdc2} kinase activity was compared to that of YAC-1 cells alone with peptide B substrate.
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15. Inhibition of protease activity was assayed with the Boc-Ala-Ala-Asp-SBzl (*2*). Fragmentin-2 cleavage of this substrate was not inhibited by peptide A. Furthermore, fragmentin-2 was not able to directly hydrolyze ^{32}P-labeled peptide A as determined by thin-layer chromatography.
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17. When held at the restrictive temperature of 39°C for 16 hours, FT210 cells showed a 75 to 85% decrease in p34^{cdc2} kinase activity with the peptide B substrate compared to cells cultured at 32°C. At the same time, the amount of p34^{cdc2} protein decreased by approximately the same extent as determined by immunoblotting. The amount and activity of p34^{cdc2} in FM3A cells were unaffected by the temperature shift.
18. After being cultured at the restrictive or permissive temperature for 6 hours, the FT210 and FM3A cells were incubated with fragmentin-2 and perforin as described (*11*) for 2 hours. The cells were then stained with 10 nM Hoechst 33258 dye and the number of apoptotic cells, which were easily identified by condensed chromatin or fragmented nuclei, were counted and expressed as a percentage of the total cell number.
19. The FT210 and FM3A cells were incubated for 6 hours at permissive and restrictive temperatures, as detailed in Fig. 4. Fragmentin-3 (0 to 1.0 μg ml^{-1}), purified as described in (*2*), was then added with a constant amount of perforin (0.36 μg ml^{-1}) for 16 hours.
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25. Cells were lysed and immunoprecipitated with antibody to p34^{cdc2} (*9*). The immunoprecipitates were subjected to electrophoresis on 12% SDS polyacrylamide gels overnight and then transferred to immobilon membranes in 39 mM glycine, 48 mM tris-HCl (pH 7.4), 0.0375% (w/v) SDS, and 20% (v/v) methanol for 1 hour. Filters were incubated with antibody to phosphotyrosine (4G10; Upstate Biotechnology Incorporated, Lake Placid, NY) or antibody to p34^{cdc2} and developed with horseradish peroxidase (HRP)–conjugated antibody to mouse immunoglobulin G (IgG) (for 4G10) or HRP-conjugated goat antibody to rabbit IgG (Boehringer Mannheim) (for antibody to p34^{cdc2}). The blots were developed with an enhanced chemiluminescence kit (ECL, Amersham).

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