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Fas-Induced Changes in cdc2 and cdk2 Kinase Activity Are Not Sufficient for Triggering Apoptosis in HUT-78 Cells

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Abstract Recent evidence suggested a role for the cell cycle dependent kinases cdc2 and cdk2 in apoptosis. An important mechanism by which many cell types could undergo apoptosis is through the activation of the Fas molecule on the cell membrane. To investigate whether Fas-induced cell death activated cdc2 and cdk2 kinases inappropriately, the human T lymphoma cells HUT-78, which express a high copy number of Fas, and two other previously characterized subclones of the same cell line which express mutant, cell death–deficient dominant-negative forms of Fas, were Fas-challenged and the changes in cdc2 and cdk2 kinase activity monitored. In both wild-type and Fas-mutated HUT-78 cells, apoptosis was associated simultaneously with decreased cdc2 and increased cdk2 activity. This association suggested that changes in cdc2 and cdk2 kinase activity are secondary events in cell death mediated by Fas. J. Cell. Biochem. 64:579–585.

Programmed cell death, known as apoptosis, is a very intensive area of investigation. Apoptosis is an important mechanism for the organism to regulate cell numbers in developing tissues, a defense mechanism for eliminating unwanted and potentially dangerous cells, such as self-reactive lymphocytes or virus-infected cells, and part of other critical biological processes [reviewed in Steller, 1994; White 1996]. Many external stimuli can initiate the apoptotic cascade of events, including cellular damage from ionizing radiation, virally infected cells, and other stimuli. However, the identification of the molecular mediators of apoptotic homeostasis is still under investigation. It has not yet been clarified whether different inducers of apoptosis have a common pathway or if they use multiple pathways with the same components.

Recently, interest has focused on the apoptotic properties of the Fas ligand (FasL) receptor (Fas) pathway [reviewed in Nagata and Goldstein, 1995]. When FasL binds to Fas, a cell surface molecule belonging to the TNF receptor family, a chain of events is triggered that ultimately leads to cell death. Fas-FasL interaction is responsible for the so-called activation-induced cell death (AICD), a process triggered by the CD3/T cell receptor complex in activated T-cells [Dhein et al., 1995; Alderson et al., 1995].

Involvement of cdc2 and cdk2 kinase complexes has been postulated among the many biochemical mediators activated during cell death. These enzymes are key components of

Abbreviations used: Ab: antibody; cdc: cell division cycle; cdk: cyclin dependent kinase; pTyr: phosphorylated tyrosine.

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the cell cycle machinery, the first acting mainly during the G2/M transition, the second acting during the S phase progression in mammalian cells [reviewed in Hunter and Pines, 1995; Nurse, 1995; MacLachlan et al., 1995]. A very simplified model of these cell-cycle-kinase complexes shows a catalytic subunit, the cdc2 molecule itself or another member of the cdc2 gene family, and a regulatory, activating subunit, the cyclin, which is cell-cycle-phase specific. If serine-threonine kinase p34cdc2 is activated prematurely, it can induce a process resembling apoptosis, termed *mitotic catastrophe*, featured by chromosome condensation and nuclear breakage. Recently, data supporting the idea that a similar phenomenon could take place during cell death induced by cytotoxic T-lymphocytes (CTLs) have been presented [Shi et al., 1994]. In addition, it has been shown that the activation-induced T-cell death in a T-cell hybridoma is regulated by M phase–specific cyclin B [Fotedar et al., 1995]. Furthermore, S phase–specific cyclin A–dependent protein kinases have been reported also to be activated during apoptosis. Experiments using drugs, such as staurosporine, caffeine, and okadaic acid [Meikrantz et al., 1994], or viral stimuli, such as the HIV-tat protein transcription factor, have shown activation of cdk2 before apoptosis [Li et al., 1995]. These results suggest that a link exists between apoptosis and unprogrammed cdk2 activation.

These results prompted us to examine whether cdc2 and cdk2 kinases are involved in the death pathway triggered by Fas. Experiments were undertaken in cell lines sensitive to Fas cross-linking including the human T-cell, the Sezary syndrome–derived HUT-78 line, and mutants of the same cell line bearing a cell-death-deficient Fas [Cifone et al., 1994, 1995; Cascino et al., 1996] in order to examine the role of cdc2 or cdk2 kinase during Fas-induced cell death. Results reported to date support the hypothesis that the activity of these enzymes is not critical for this form of apoptosis.

MATERIALS AND METHODS

Cell Line, Induction, and Assessment of Apoptosis

The human T-cell lymphoma, Sezary syndrome–derived, HUT-78, or Fas-mutant clones of the same cell line were used for the experiments. Cells were grown in RPMI 10% fetal calf serum. Fas stimulation was done on asynchronously growing cells by adding anti-Fas antibody at a concentration of 500 ng/ml, considered a saturating condition, and at a cellular density of 1×10^6 cells/ml. The same conditions were used for the anti-IgM control stimulation. DNA fragmentation was detected as reported [Cifone et al., 1994].

Antibodies

Anti-Fas and antiphosphotyrosine mouse monoclonal Ab 4G10 antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Human anti-cdc2 and anti-cdk2 antisera were raised against a peptide corresponding with amino acid residues mapping at the carboxy terminus (ADL4 and ADL5). Cyclin-A and cyclin-B antisera were obtained by immunizing rabbits with bacterially expressed glutathione S-transferase (GST)–cyclin A and GST–cyclin B fusion proteins (ADL6 and ADL7).

Western Blots

Cell lysates were prepared by resuspending pelleted cells in 200 µl lysis buffer (50 mM Tris, 5 mM EDTA, 250 mM Nacl, 50 mM NaF, 0.1% Triton, 0.1 mM Na3VO4, plus protease inhibitors). Protein (50 µg) was run on a 10% polyacrylamide gel. Proteins within the polyacrylamide gel were transferred to a PVDF membrane (Millipore, Bedford, MA) in CAPS buffer (10 mM CAPS, 20% methanol, pH 11). The membrane was blocked with 5% milk in TBS-T buffer (2 mM Tris, 13.7 mM NaCl, 0.1% Tween-20, pH 7.6) and then washed in TBS-T. Primary antibody was incubated with the membrane in 3% milk and then washed in TBS-T. The membrane was then incubated with a rabbit antimouse Ab coupled with horseradish peroxidase and washed in TBS-T. The presence of secondary antibody bound to the membrane was detected using the ECL system (Dupont-NEN, Boston, MA).

Kinase Assays

Cell lysates were prepared by resuspending pelleted cells in 200 µl lysis buffer (50 mM Tris, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton, 0.1 mM Na3VO4, plus protease inhibitors). An equal amount of protein for each fraction was immunoprecipitated with specific Ab and tested for the presence of histone H1 kinase activity. Protein kinase assays were performed as described [Giordano et al., 1989]. Kinase assays were repeated at least three

times, giving an interassay standard deviation within 10% after normalization for protein amount.

RESULTS

Fas-induced apoptosis was studied in HUT-78 cells because they are extremely sensitive to Fas cross-linking. The cell death program was activated by adding anti-Fas Ab to the cells. DNA degradation, assessed by propidium iodide staining and by FACS analysis, could be detected after 6 h [Cifone et al., 1994, 1995; Cascino et al., 1996] (data not shown).

We first asked whether the protein levels of cdc2, cdk2, cyclin A, and cyclin B were modulated throughout the experiments. A Western blot performed on cell lysates demonstrated that these proteins maintained constant levels during the first 7 h of Fas stimulation (Fig. 1).

The activity of the kinases associated with p34cdc2, p33cdk2, cyclin B1, and cyclin A was measured by immunoprecipitation with specific antibodies, followed by a kinase assay on histone H1, an exogenous conventional substrate. In this experiment, cells were harvested 0, 1, 3, 5, and 7 h after Fas stimulation. The results reported as histograms in Figure 2 show that, after 1 h, the activity of p34cdc2 was reduced in comparison with time 0 and became approximately one-fifth at 5 h. Interestingly, p33cdk2 activity increased simultaneously and significantly with the decrease in p34cdc2 activity. Cyclin B1– and cyclin A–associated kinase activity followed a pattern similar to that seen with the p34cdc2- and p33cdk2-associated kinases. Control experiments were done by stimulating HUT-78 cells with an unrelated IgM, which gave no change in cdc2 and cdk2 kinase activity (data not shown).

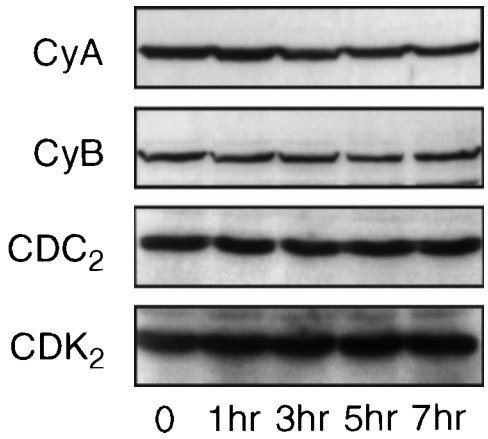

Fig. 1. Western blot showing levels of cyclin A, cyclin B, p34cdc2, and p33cdk2 during the first 7 h of Fas stimulation in wild-type HUT-78 cells.

In contrast to reports which showed activation of cdc2 during apoptosis [Shi et al., 1994; Fotedar et al., 1995], our data suggest that p34cdc2 activity decreases significantly in HUT-78 cells after Fas stimulation. In our experiments, the reduced activity of p34cdc2 has been found to be independent of the amount of cyclin B or p34cdc2. This observation could be attributed to the fact that the activity of p34cdc2 can change by Tyr 5 phosphorylation. Therefore, we measured, by Western blot, the level of Tyr phosphorylation of p34cdc2 before and after Fas stimulation using an antiphosphotyrosine Ab on an anti-p34cdc2 immunoprecipitate after the first hour. Figure 3 shows an increase in pTyr phosphorylation levels consistent with the down modulation of p34cdc2 activity after the first hour of Fas challenging.

Having assessed the decrease in p34cdc2 activity and the increase in p33cdk2 kinase activity in wild-type HUT-78 cells, we asked if the Fas-induced cdk2 kinase activity changes represented an obligate crossroad for apoptosis. In order to answer this question, we took advantage of two previously characterized subclones of HUT-78 cells (clone B1 and clone B15) bearing Fas-mutant molecules. These Fas molecules can bind FasL but lack a functionally active intracytoplasmic death domain, so they are unable to transmit the death signal but do retain the ability to activate other signal transduction pathways [Cifone et al., 1994, 1995]. We reasoned that if Fas activation was able to induce the same changes in cdk2 kinase activity in these cells as in wild-type HUT-78 cells, this would suggest that an unscheduled increase in cdk2 kinase per se is not sufficient to trigger cell death. To prove this, cells were harvested 0, 1, 3, and 5 h after Fas stimulation, and kinase activity of p34cdc2 and p33cdk2 was performed. The results reported as histograms in Figure 4 show that the modifications of a stimulations of cdk2 activity and an inhibition of cdc2 activity are similar to those of the wild-type HUT-78 cells. Stimulation of the same clones with a control IgM left the activity bound to cdc2 and cdk2 unaltered (data not shown). In addition, the levels of cyclin B, cyclin A, p34cdc2, and p33cdk2 were constant in mutant clones (data not shown).

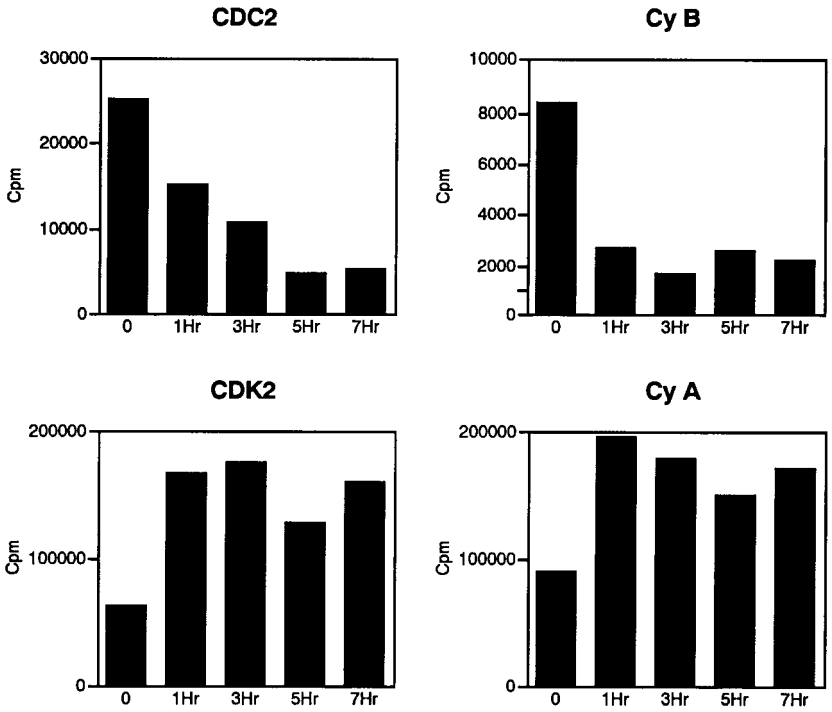

Fig. 2. Time course of p34cdc2, cyclin B-, p33cdk2, and cyclin A–associated kinase activities during the first 7 h of Fas stimulation in wild-type HUT-78 cells. The associated activity is expressed as counts per million on the ordinate and time in hours on the abscissa.

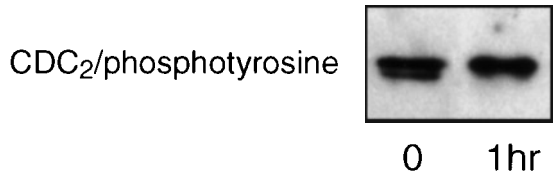

Fig. 3. p34cdc2 tyrosine phosphorylation after the first hour of Fas-challenging. Immunoprecipitates of p34cdc2 were blotted with anti-phosphotyrosine monoclonal antibody.

FACS analysis was performed to rule out the possibility that changes in cdk2 and cdc2 were due to modification of the percentage of cell cycle phases (Fig. 5). No significant changes could be detected after 4 h of anti-IgM unrelated stimulation (Fig. 5a, c) and fas stimulation (Fig. 5b, d) in wild-type HUT-78 and in clone B1 cells. Therefore, changes in cdc2 and cdk2 activities are unrelated to any relevant changes in the cell cycle.

DISCUSSION

The cdc2 and cdk2 kinase complexes are made up of a catalytic subunit p34cdc2 and p33cdk2 that associates at specific phases of the cell cycle with the regulatory subunits, the B cyclins (B1 and B2) and cyclin A, among the others. B cyclins, in particular B1, regulate the M phase in eukaryotic cells. In addition to the simple physical interaction between cyclin B and p34cdc2, cdc2 kinase complex activity is regulated also by phosphorylation/dephosphorylation events. Phosphorylation of p34cdc2 in tyrosine 15 by the human homologue of the *S. pombe* gene wee1 has been shown to be critical for the inhibition of its kinase activity. The dephosphorylation at the same residue increases the activity similar to the control of G2/M phase transition in *S. pombe*. Cyclin A, on the other hand, has a preferential partner in p33cdk2, and activity of the cyclin A–cdk2 complex regulates the progression of the cell throughout the S phase.

In recent reports, modulation of the kinase activity of both cdc2 and cdk2 complexes has been associated with apoptotic events, triggered by many stimuli. In our experimental system, a fivefold decrease of cdc2 kinase activity is detected after Fas-challenging. Our results, therefore, tend to support a recent view that cdc2 is not part of the apoptotic machinery [Martin et al., 1995]. They also confirm the findings reported by Meikranz et al. [1994] and

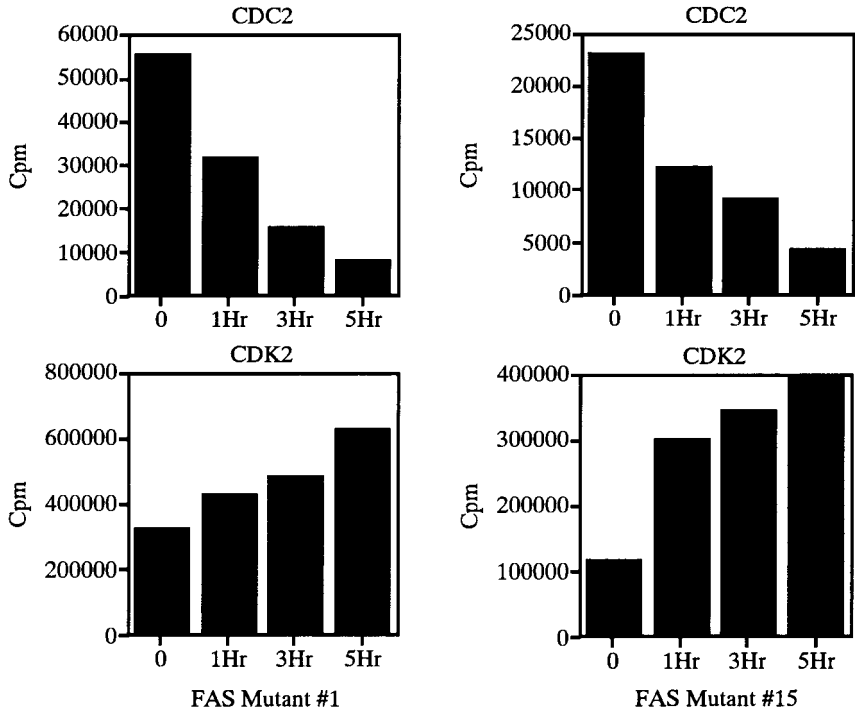

Fig. 4. Time course of p34cdc2- and p33cdk2-associated kinase activities during the first 5 h of Fas stimulation in Fas-mutant HUT-78 clones 1 and 15. The associated activity is expressed as counts per million on the ordinate and time in hours on the abscissa.

Li et al. [1995] in that there is an activation of cdk2 during apoptosis. The phenomenon of an activation of cdk2 in Fas-mutated clones, though, tends to exclude the possibility that changes in cdk2 activity are essential for Fas-mediated apoptosis, at least in the cell line examined here.

It is possible that the discrepancy between our data and data reported by Shi et al. [1994] may be the result of the difference in experimental conditions. Shi et al. [1994] studied apoptosis induced by granzyme B and perforin, proteins released by cytotoxic T lymphocytes and natural killer cells on the target cells. In addition, cells were permeabilized, and a synthetic substrate peptide of p34cdc2 kinase was added to the medium in order to block its activity. One possibility could be that there may have been an unspecific block of serine-threonine kinases other than p34cdc2 involved in apoptosis by administering the synthetic substrate for cdc2.

Our experiments show that the decrease in cdc2 activity is not because of cyclin B or p34cdc2 degradation but rather because of tyr-phosphorylation of p34cdc2. Fas-induced p34cdc2 inactivation, then, is at least partially dependent upon its specific phosphorylation. It would be of interest in future experiments to learn how enzymes regulating the function of p34cdc2, like wee1, cdc25, or cdk inhibitors are affected by the signaling triggered by Fas-FasL interaction. Recently, the apoptosis induced by the myc oncogene was shown also to be independent of cdc2 and cdk2 activity. In fact, blocking the activity of those kinases with p21 was not able to prevent cell death induced by myc [M. Eilers, EMBO J., in press, personal communication]. Another intriguing aspect of our results is the differential regulation of p34cdc2 and p33cdk2 during Fas apoptosis.

A newly cloned member of the cdc2 gene family, PITSLRE, has been recently associated positively with apoptosis [Lahti et al., 1995]. Conversely, the activity of another member of the cdc2 gene family cloned in our lab, PITALRE [Graña et al., 1994], which is closely related to PITSLRE is not increased during apoptosis mediated by Fas (data not shown). It seems clear, therefore, that differential changes in activity of the various members of the cdc2 gene family occur during different types of apoptosis. Our data show that changes of activity in cdc2 and cdk2 are not sufficient for mediating cell death. We speculate that the modulation of these activi-

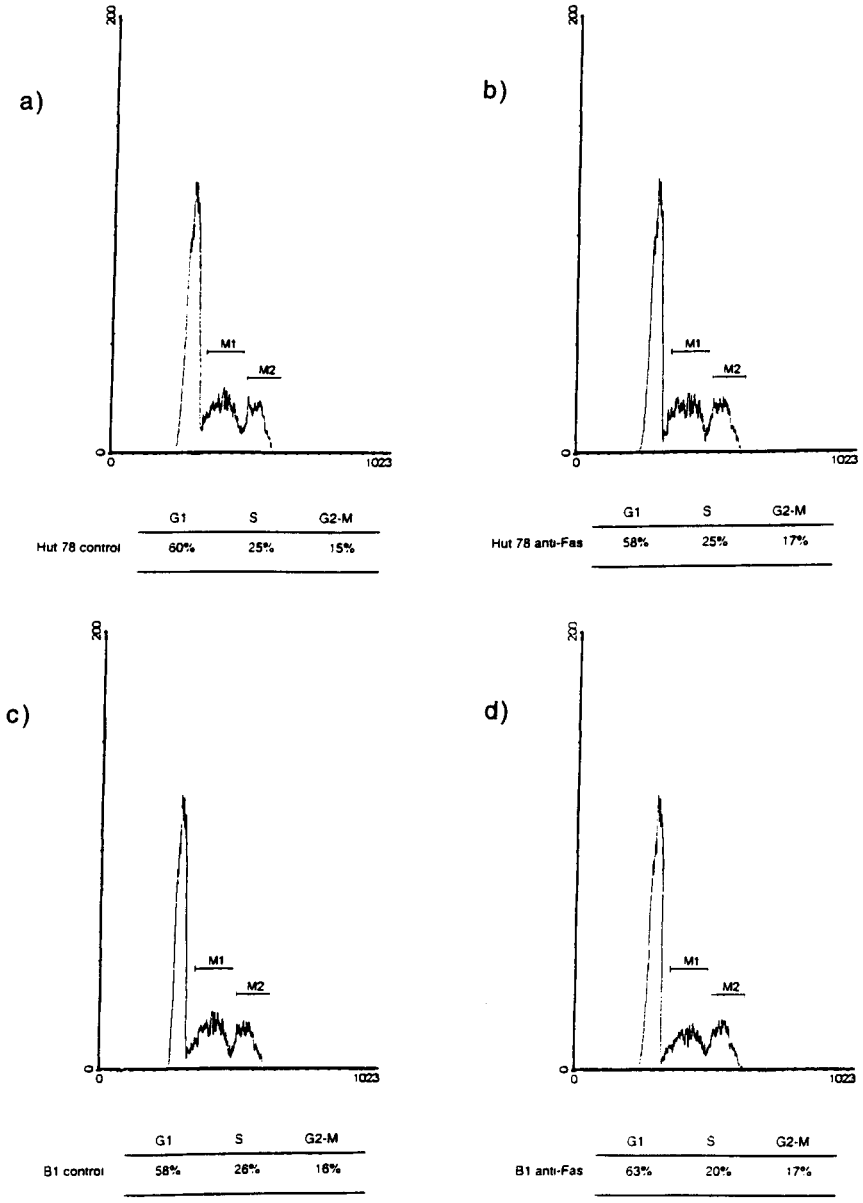

Fig. 5. FACS analysis of HUT-78 wild-type and Fas-mutant HUT-78 clones B1 cells after 4 h of anti-IgM unrelated stimulation (a, c) and Fas stimulation (b, d).

ties is a secondary, nonessential phenomenon of the Fas signal transduction cascade that causes cell suicide.

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