Suppression of Apoptosis by Dominant Negative Mutants of Cyclin-dependent Protein Kinases*

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In many cell types, position in the cell cycle appears to play a role in determining susceptibility to apoptosis (programmed cell death), and expression of various cyclins and activation of cyclin-dependent kinases (CDKs) have been shown to correlate with the onset of apoptosis in a number of experimental systems. To assess the role of CDK-mediated cell cycle events in apoptosis, we have expressed CDK dominant negative mutants in human HeLa cells. Dominant negative mutants of CDC2, CDK2, and CDK3 each suppressed apoptosis induced by both staurosporine and tumor necrosis factor α, whereas a dominant negative mutant of CDK5 was without effect. Like CDC2 and CDK2, CDK3 was shown to form a complex with cyclin A in vivo. CDK5 did not bind cyclin A to any detectable extent. Overexpression of wild type CDC2, CDK2, CDK3, or cyclin A (but not cyclin B) markedly elevated the incidence of apoptosis in BCL-2−/− cells, which otherwise fail to respond to these agents. These results help identify cell cycle events that are also important for efficient apoptosis.

A number of studies suggest that apoptosis is linked with cell cycle events (1). Within the organism, apoptosis is found primarily in proliferating tissues (2, 3) and is associated with induction of proliferation-associated genes (4–6). Quiessent fibroblasts are resistant to cytotoxic lymphocyte-induced apoptosis (7), and resting peripheral blood T cells are resistant to activation-induced death by ligation of the T cell receptor (8). Blocking proliferation with c-myc antisense oligonucleotides blocks activation-induced death of T cells (9), and E1A mutants that are defective in induction of DNA synthesis also fail to induce apoptosis in susceptible cells (10). Cells undergoing apoptosis frequently seem to do so from late in G1 or early in S phase whether the stimulus is ligation of antigen receptors (11, 12), growth factor deprivation (5, 13, 14), or restoration of wild type p53 function (15, 16). Arrest in G0 or early G1 suppresses apoptosis in response to a wide range of agents (9, 14–20), whereas arrest late in G1, or in S phase can accelerate or potentiate apoptosis (15, 16, 18, 19). These data imply the existence of molecules present in late G1 and S phase whose activities facilitate the execution of apoptosis.

Cell proliferation is regulated by cell cycle-specific synthesis of cyclins and activation of cyclin-dependent kinases (CDKs).1 Response to growth factors and passage through G1 is mediated by D-type cyclins, whereas control of entry into S phase (commencement of DNA replication) is regulated by protein kinases associated with cyclin E and cyclin A (see reviews in Refs. 21 and 22). Cyclin D- and cyclin E-associated kinases phosphorylate pRB, the protein product of the retinoblastoma susceptibility gene, allowing E2F-dependent transcription of genes whose products are required for DNA replication (e.g. DNA polymerase α), whereas cyclin A-associated kinases participate in the phosphorylation of substrates associated with formation of the replication fork (e.g. replication factor A (21, 22)). We have shown previously that arrest of HeLa cells in late G1 or early S phase greatly potentiates the apoptosis-inducing ability of a variety of agents (19). Induction of apoptosis was uniformly associated with activation of cyclin A-dependent kinases but not activity associated with cyclins E or B (19). These results suggested that cyclin A might act as a cell cycle-dependent facilitator of apoptosis. To define at a molecular level cell cycle events that modify the ability to undergo apoptosis, wild type and dominant negative mutants of CDKs (23) were transiently expressed in HeLa cells prior to exposure to apoptosis-inducing agents. We report that dominant negative mutants of CDC2, CDK2, and CDK3 suppressed apoptosis in response to both staurosporine and TNF-α. In addition, overexpression of the wild type form of these kinases or cyclin A circumvented the anti-apoptosis activity of the oncogene BCL-2.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmids—Human HeLa cells derived from a cervical carcinoma were grown in monolayer culture as described previously (10). A HeLa line (clone HB14), which stably overexpresses human BCL-2 under control of the SV40 enhancer and promoter (24), was maintained in G418 as described previously (19). Wild type CDK-expressing plasmids under control of the CMV promoter (pCMVcdc2, pCMVcdk2, pCMVcdk3, and pCMVcdk5) and dominant negative CDK-expressing plasmids (pCMVcdk2-dn, pCMVcdk2-dn, pCMVcdk3-dn, and pCMVcdk5-dn) with or without the hemagglutinin epitope (HA) tag were a gift from Drs. Sander van den Heuvel and Ed Harlow (23) (Massachusetts General Hospital). The lacZ-expressing plasmid pCMVβ-gal was a gift of Dr. Arthur Lee (Harvard School of Public Health), and the cyclin A-expressing plasmid pCMVCycA was from Dr. Phil Hinds (Harvard Medical School) (25). A plasmid expressing human cyclin B containing a myc-derived epitope tag was a gift of Dr. Frank McKeen (Harvard Medical School) (26).

Transfection and Analysis of Transfected Cells—2 × 10^5 HeLa cells grown on glass coverslips were co-transfected with 0.4 μg pCMVβ-gal (lacZ) and 2 μg of either pCMVcdk2-dn, pCMVcdk3-dn, pCMVcdk3-dn, or pCMVcdk5-dn-HA using LipofectACE (Life Technologies, Inc.) according to the manufacturer’s instructions. For the experiments shown in Fig. 4, 10 μg of wild type plasmid was included along with 1

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1 The abbreviations used are: CDK, cyclin-dependent kinase; dn, dominant negative mutant; TNF-α, tumor necrosis factor α; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; HA, hemagglutinin epitope.
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RESULTS

Inhibition of Apoptosis by CDK Dominant Negative Mutants—Apoptosis was induced in HeLa cells by the addition of either TNF-α and cycloheximide or staurosporine and cycloheximide. These treatments were selected because they rapidly induce apoptosis in numerous cell types, both primary and transformed (29–31), and are thus believed to activate elements of a death program common to all cells (31, 32). To assess the role of CDKs in apoptosis induced by these agents, CDK-dns were transfected into cells 48 h prior to induction of apoptosis. When expressed under control of a strong promoter, the catalytically inactive CDK-dns compete with wild type CDKs for cyclin binding and thus act as competitive inhibitors of CDK activity (23). Following induction of apoptosis, HeLa cells cotransfected with CDK-dns and a lacZ marker plasmid were fixed and stained for β-galactosidase activity with a chromogenic substrate (Fig. 1, a and b) or for indirect immunofluorescence expression by indirect immunofluorescence (Fig. 1, c and d), thereby allowing microscopic determination of the apoptosis frequency among lacZ+ cells. Alternatively, expression of HA-tagged CDK mutants (CDK-dn-HA) permitted direct detection of CDK-dn-transfected cells by indirect immunofluorescence with a hemagglutinin-specific monoclonal antibody (Fig. 1, e and f). Note that the presence of transfection markers for cdk2-dn and cdk3-dn correlates with the absence of apoptosis in the repres
CDK3, which, like CDK2, is required for the G1/S phase transition, has not been described previously. However, as shown in Fig. 3, cdk3-dn-HA, like cdk2-dn-HA, was co-precipitated with antibodies to cyclin A from extracts of cells overexpressing these constructs. Cdk5-dn-HA failed to associate with cyclin A.

Cdk3 Associates with Cyclin A in Vivo—A cyclin partner for CDK3, which, like CDK2, is required for the G1/S phase transition, has not been described previously. However, as shown in Fig. 3, cdk3-dn-HA, like cdk2-dn-HA, was co-precipitated with antibodies to cyclin A from extracts of cells overexpressing these constructs. Cdk5-dn-HA failed to associate with cyclin A.

Co-transfection of cdk3-dn-HA with a 10-fold excess of wild type CDK3 lacking the epitope tag blocked the association of cyclin A with cdk3-dn-HA, indicating that wild type CDK3 can compete with cdk3-dn-HA for cyclin A binding. Control cyclin A immunoprecipitations from cells overexpressing either wild type CDC2 or wild type CDK2 displayed no nonspecific HA-reactive species. These data show that CDK3, when overexpressed, will bind cyclin A in vivo and imply that at least part of its activity in this model system may be cyclin A-dependent.

Although the subcellular localization of CDK3 is unknown, CDC2, CDK2, and cyclin A reside in both the nucleus and cytoplasm of HeLa cells (19, 34, 42). Because apoptotic events within these compartments can occur independently (43, 44), it is noteworthy that inhibition of CDKs blocked both the cytoplasmic (cell rounding and surface blebbing; Fig. 1a) and nuclear events (chromatin condensation; Fig. 1, c and e) associated with apoptosis. We note that complete inhibition of apoptosis is not seen with any individual CDK-dn or with any combination of CDK-dns. This may be due to the elevated levels of cyclins that are found in human papillomavirus-positive cells (45) (making competition for positive regulatory factors by CDK-dns more difficult in HeLa cells), to variations in the level of CDK-dn protein expressed transiently in each cell, or to additional apoptotic pathways that are independent of CDK activity.

Cyclin A or Wild Type CDK Expression Reverses the Effect of CDK-dns—Although cdk5-dn provides a useful comparison for the effects of the other CDK-dns, the best control is to counter the effect of a mutant CDK by co-expression of the wild type CDK or its cyclin partner (23). Thus, wild type CDKs and cyclin A were co-transfected with the CDK-dns to see if inhibition of apoptosis could be reversed. As shown in Fig. 4, co-expression of either cyclin A or the wild type CDKs was able to overcome the inhibitory effect on apoptosis of each CDK-dn. Among the wild type CDKs, CDC2 was least effective, whereas CDK2 and CDK3 were similarly effective at blocking inhibition by all three CDK-dns. Inhibition was blocked most effectively by cyclin A co-transfection. Indeed, in some cases cyclin A was able to drive cells to apoptosis frequencies higher than those seen in control cells transfected with lacZ alone. These results suggest redundant functions of CDC2, CDK2, and CDK3 in apoptosis.

The ability of any one dominant negative mutant to inhibit apoptosis is likely due to competition between the mutant and the three wild type kinases for the same CDK activating component(s).

Cyclin A or CDK Overexpression Can Promote Apoptosis in a BCL-2-Background—Although the above experiments indicate that inhibition of certain CDKs suppresses apoptosis, it is possible that this suppression is accomplished through an indirect mechanism. For example, Cdk2-dn and Cdk3-dn mutants are known to prevent entry of cells into S phase (23), and a biochemical pathway required for apoptosis may be active only during this phase of the cell cycle. If, however, overexpression of cyclin A or wild type CDKs in asynchronously growing cell populations increases apoptosis in response to agents such as TNF-α, a closer relationship between these proteins and apoptosis may be implied, particularly if increases occur in the absence of gross cell cycle perturbation. To address this issue, we overexpressed wild type CDKs and cyclin A in asynchronously growing HB14 cells, a HeLa cell line that stably overexpresses the BCL-2 oncogene and is resistant to staurosporine and TNF-α-induced apoptosis (19). When HB14 cells were transiently transfected with either wild type CDC2, CDK2,
CDK3, or cyclin A, the levels of TNF-α-induced apoptosis increased from approximately 2-9% in control transfections to as high as 70%, a level approaching that seen in the BCL-2-deficient parental HeLa cells (Fig. 5). The proportion of cells in S phase was not significantly altered in cells expressing cyclin A (32%) when compared with nonexpressing cells in the same dish (34%), as determined by autoradiography of cells labeled with [3H]thymidine and stained for β-galactosidase activity, indicating that the increase in apoptosis was not due to an increase in the number of cells in S phase. In contrast to cyclin A, expression of cyclin B in HB14 cells had no effect on apoptosis. Consistent with the earlier CDK-dn experiments, CDC2 was less effective than CDK2, CDK3, and cyclin A at enhancing apoptosis in the presence of BCL-2. Similar results were obtained when apoptosis was induced by staurosporine or 6-dimethylaminopurine and when a second independent BCL-2‡ clone was used (data not shown). Only modest increases in apoptosis were obtained in HeLa cells, indicating that the level of expression of these genes is not rate-limiting for apoptosis in the parental cells. Minimal toxicity was seen when CDKs or cyclin A were overexpressed in the absence of TNF-α, in agreement with the findings of others for CDC2 and CDK2 (23, 26, 46).

DISCUSSION

We have shown that three different dominant negative CDK mutants are capable of blocking apoptosis in response to two general inducers of apoptosis, TNF-α and staurosporine. The inhibitory effect could be overcome by co-expression of cyclin A or by the wild type form of any of the other three wild type CDKs. This functional redundancy suggests that the dominant negative mutants exert their effects via competition for a common activating factor or factors, a conclusion further supported by the finding that co-transfection of two or more CDK-dns was no more effective than single CDK-dns for suppressing apoptosis. It is possible that this shared activating factor is cyclin A, because all three mutants can bind this cyclin.

These data are consistent with other published results suggesting that events downstream of cyclin A-dependent kinase activation are important for apoptosis. For example, cyclin A is transcribed in response to two inducers of apoptosis, c-myc and E1A (47–50). Elevated cyclin A expression from an inducible promoter can induce apoptosis in serum-starved fibroblasts (48), and cyclin A-dependent kinases are specifically activated when apoptosis is induced in T cell lines by human immunodeficiency virus tat (51) and in HeLa cells by a variety of physiological and pharmacological agents (19). In target cells killed by granulysin B, CDC2 activity was increased (52), including activity associated with cyclin A. Use of a temperature-sensitive CDC2 mutant significantly reduced apoptosis in this system (52), and transfection of target cells with wee1, a tyrosine kinase that phosphorylates and inactivates CDKs, also inhibited apoptosis (53).

Results from experiments performed with dominant negative CDK mutants must be interpreted cautiously. Such mutants may be expected to exert pleiotropic effects due to their interference with the cell cycle. Although we have shown that overexpression of cyclin A or its wild type catalytic partners can drive apoptosis to high levels in BCL-2‡ cells in the absence of gross cell cycle perturbations, these data do not demonstrate a direct involvement of cyclins or CDKs in apoptosis. The biochemical pathways that emanate from activation of any cyclin-CDK complex are most certainly complex, wide-ranging, and intricately involved in many different facets of cell proliferation. However, these findings do identify cell cycle components

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\[ \text{CDK complex} \]
whose activity, when modulated, can promote or impede apoptosis. The existence of such components supports the notion that specific cell cycle events must be completed before apoptosis can occur in an efficient manner and suggests that cell cycle regulatory proteins might be useful therapeutic targets for manipulating apoptosis.

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