Cdc2 and Cdk2 Kinase Activated by Transforming Growth Factor-β1 Trigger Apoptosis through the Phosphorylation of Retinoblastoma Protein in FaO Hepatoma Cells*

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The signaling pathway leading to TGF-β1-induced apoptosis was investigated using a TGF-β1-sensitive hepatoma cell line, FaO. Cell cycle analysis demonstrated that the accumulation of apoptotic cells was preceded by a progressive decrease of the cell population in the G1 phase concomitant with a slight increase of the cell population in the G2/M phase in response to TGF-β1. TGF-β1 induced a transient increase in the expression of Cdc2, cyclin A, cyclin B, and cyclin D1 at an early phase of apoptosis. During TGF-β1-induced apoptosis, the transient increase in cyclin-dependent kinase (Cdk) activities coincides with a dramatic increase in the hyperphosphorylated forms of RB. Treatment with roscovitine or olomoucine, inhibitors of Cdc2 and Cdk2, blocked TGF-β1-induced apoptosis by inhibiting RB phosphorylation. Overexpression of Bcl-2 or adenovirus E1B 19K suppressed TGF-β1-induced apoptosis by blocking the induction of Cdc2 mRNA and the subsequent activation of Cdc2 kinase, whereas activation of Cdk2 was not affected, suggesting that Cdk2 plays a more critical role in TGF-β1-induced apoptosis. In conclusion, we present the evidence that Cdc2 and Cdk2 kinase activity transiently induced by TGF-β1 phosphorylates RB as a physiological target in FaO cells and that RB hyperphosphorylation may trigger abrupt cell cycle progression, leading to irreversible cell death.

TGF-β1 is a multifunctional cytokine playing critical roles in many cellular processes, including cellular growth, differentiation, and morphogenesis (1, 2). Although TGF-β1 has classically been shown to arrest growth at the G1 phase of the cell cycle (3), its role has more recently been demonstrated as a key regulator in the orchestration of cell survival or death. However, the precise mechanism underlying the TGF-β1-induced apoptotic response in various cell types remains unclear. The overexpression of Bcl-2 has been shown to correlate with the onset of apoptosis in many experimental systems (33–43). However, the physiological target(s) of these kinases during apoptosis have not been elucidated. The oncogene product Bcl-2 effectively protects cells from apoptotic cell death (41). Recently, the inhibition of cell death by Bcl-2 has been linked to the slowdown of cell cycle progression (33–43). However, the detailed action mechanism of Bcl-2 for death-sparing activity still remains to be clarified.

We demonstrate here that TGF-β1 induces apoptosis in FaO rat hepatoma cells through the distinctive controlling mechanism of cell cycle components in FaO cells, which is contradic-
tory to the mechanism for TGF-β1-induced G1 cell cycle arrest that was previously reported. In addition, we present the evidence for the first time that RB is a functional substrate of Cdk2 and Cdk2 kinase transiently activated by the signal of TGF-β1 to trigger apoptosis. Moreover, we show that overexpression of Bcl-2 protein protects the cells from TGF-β1-induced cell death by blocking the induction of Cdc2 mRNA and the subsequent activation of Cdc2 kinase to phosphorylate RB.

MATERIALS AND METHODS

Reagents—Antibodies against Cdk2, Cdk4, Cdk6, cyclin B, and cyclin D1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against p27 was purchased from Transduction Laboratories (Lexington, KY). Antibodies against p21, Cdk2, and cyclin A were from Oncogene Research Products (Cambridge, MA). Antibody against phospho-RB-epitope specific RB was provided by New England Biolabs, Inc. (Beverly, MA). Histone H1 was purchased from Sigma. Recombinant RB-N terminus fusion protein containing RB residue 181–400 and RB-C terminus fusion protein containing 701–928 were obtained from New England Biolabs (Beverly, MA). Roscovitine, olomoucine, and Pancorbin were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). [γ-32P]ATP and enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech, and the protein assay reagents were from Bio-Rad. All other reagents and compounds were analytical grades.

Cell Culture—FaO rat hepatoma cells were cultured in 10% fetal bovine serum. Cells were split 1 day before TGF-β1 treatment. TGF-β1 (5 ng/ml) was added to 70–80% confluent cells. Recombinant TGF-β1 was used to induce cell death by blocking the induction of Cdc2 mRNA and the subsequent activation of Cdc2 kinase to phosphorylate RB.

Nuclear Staining with Hoechst 33258—FaO cells were split onto coverslips and incubated for 1 day prior to TGF-β1 treatment. Cells were treated with TGF-β1 for the indicated times and fixed with 4% paraformaldehyde (pH 7.4) for 10 min, washed with phosphate-buffered saline (PBS) three times, and then incubated for 10 min in 10 μg/ml Hoechst 33258 in PBS. The change of nuclear morphologies was examined by fluorescence microscopy.

Immunoblotting—Cells were harvested, and fragmented DNA was isolated by phenol–chloroform extraction. Gels were stained with Coomassie Blue staining solution and dried. The extent of phosphorylation was measured by liquid scintillation counting of the gel slices using the modality.

Establishment of Bcl-2- or E1B 19K-overexpressing FaO Stable Cell Line—FaO cells were transfected with a mammalian expression vector containing full-length bcl-2 cDNA and with a vector containing full-length E1B 19K cDNA (Ref. 44; kindly provided by Dr. Zarchuck, National Institutes of Health, Bethesda, MD) and selected with changes in drug concentrations for 4–6 weeks. FaO sublines stably transfected with an empty vector were used as a control. The expression of the transfected gene products in the selected cell lines was confirmed by Northern blot analysis.

RESULTS

TGF-β1 Induces Apoptosis in the Rat Hepatoma Cell FaO—TGF-β1 is a potent apoptotic inducer in hepatocytes (4–6). FaO is a well differentiated hepatoma cell line that is very sensitive to the apoptotic effect of TGF-β1, even in complete media containing 10% fetal bovine serum, as we previously reported (12, 45). TGF-β1 induces cell death in FaO cells with morphological changes such as cell shrinkage and cytoplasmic blebbing, which are characteristic of progression of apoptosis (Fig. 1A). In addition, chromatin has a characteristic condensed and fragmented appearance following Hoechst 33258 staining of cells treated with TGF-β1 for 12 h. Moreover, TGF-β1 induced a clear 180–200-base pair internucleosomal DNA cleavage after TGF-β1 treatment (Fig. 1B).

Regulatory Mechanism of Cell Cycle Components during TGF-β1-induced Apoptosis Does Not Overlap with TGF-β1-induced Growth Arrest Pathway—Next, we explored the possible differences between the TGF-β1-mediated apoptotic pathway and the TGF-β1-induced G1 cell cycle arrest pathway. First, we examined whether TGF-β1-induced G1 cell cycle arrest is a prerequisite for apoptosis. Interestingly, cell cycle analysis demonstrated that the induction of apoptosis by TGF-β1 was not preceded by G1 cell cycle arrest. Rather, a progressive increase of the G1 phase population and an increase in the cells in G0/M phase were observed prior to TGF-β1-induced apoptosis (Fig. 2). These results suggest that loss of G1 cell cycle arrest may be implicated in the TGF-β1-induced apoptotic mechanism.

We then attempted to study whether these changes of the cell cycle profile during TGF-β1-induced apoptosis can be explained in terms of the changes in expression of the cell cycle...
regulatory components. We were interested in whether some cell cycle regulatory components are shared between the TGF-β1-induced apoptotic pathway and the TGF-β1 growth inhibitory pathway, or if the regulatory mechanisms controlling cell cycle progression are completely separate for the two pathways. We explored the expression of cyclins, Cdkks, and Cdk inhibitors, which have previously been implicated in TGF-β1-mediated cell cycle arrest during TGF-β1-induced apoptosis (Fig. 3A). The protein levels of Cdk2, Cdk4, Cdk6, cyclin E, and p27 were not significantly altered. The levels of proteins such as cyclin A, cyclin B, and cyclin D1, which are involved in the progression of the cell cycle, showed a gradual rise with a sustained peak at around 8 h after TGF-β1 treatment, and then a subsequent decline. Upon TGF-β1 treatment, the Cdc2 protein was induced at 4 h, reached its peak at 8 h, and then decreased to its previous level by 24 h. Several recent papers suggest that tyrosine phosphorylation of Cdc2 (46, 47) is associated with the inactivation of Cdc2 kinase activity. However, no significant change in the tyrosine phosphorylation level of Cdc2 was observed during TGF-β1-induced apoptosis (data not shown).

To investigate whether the changes in protein levels of some cell cycle components are caused by the changes in mRNA levels, Northern blot analysis was performed after TGF-β1 treatment (Fig. 3B). Levels of Cdc2, cyclin A, and cyclin B mRNAs were dramatically induced at 2 h after TGF-β1 treatment and then showed a gradual decline. The level of cyclin D1 mRNA was induced at 2 h after TGF-β1 treatment and was sustained for at least 12 h. The mRNA level of p21 was quite abundant, but was slightly induced at 2 h after TGF-β1 treatment, and then gradually decreased. In contrast, Cdk2, cyclin E, and p27 did not demonstrate any significant changes in mRNA levels during the progression of TGF-β1-induced apoptosis. These results suggest that the expressions of cyclins, Cdkks, or Cdk inhibitors involved in TGF-β1-induced growth inhibition are regulated differently during TGF-β1-induced apoptosis, except for a slight induction in p21.

Cdkks Are Transiently Activated during TGF-β1-induced Apoptosis—Cyclin-dependent kinases (Cdkks) are regulated by their association with cyclins; cyclin A (Cdc2, Cdk2), B (Cdc2),
D (Cdk2, Cdk4, and Cdk6), and E (Cdk2) (13, 14). Since the expression of Cdc2 and cyclins including cyclin A, B, and D1 was changed during TGF-β1-induced apoptosis, we investigated whether the activities of cyclin-dependent kinases are affected by the changes in the expression of Cdc2 itself or the associated cyclins. The kinase activities associated with Cdc2 and Cdk2 were measured by immunoprecipitation with respective antibodies, followed by a kinase assay on histone H1 as an exogenous conventional substrate (Fig. 4A). Cdc2 kinase activity showed an approximately 4.4-fold rise after 8 h of treatment with TGF-β1, and a gradual decline after a peak in parallel with the change in the Cdc2 protein level as shown in Fig. 3A. Interestingly, Cdk2-associated histone H1 kinase activity was also markedly increased, despite the fact that the Cdk2 protein level was not changed. Western blot analysis of immunoprecipitated Cdk2 revealed the enhanced association of cyclin D1 with Cdk2 at the peak time of Cdk2 kinase activity (Fig. 4B), suggesting that increased cyclin D1 expression contributes to the increase of Cdk2 kinase activity. Recent studies have reported that histone H1 is a poor substrate for Cdk4 and Cdk6 (48). In consistent with their results, not only Cdk4 but also Cdk6 kinase activity on histone H1 was barely detected before or after TGF-β1 treatment in our study (data not shown). Therefore, the changes in Cdk4 and Cdk6 kinase activity were analyzed again using the RB-C terminus fusion protein containing residues 701–928, which is a commonly used substrate for Cdk4 or Cdk6 kinase assay. However, activation of Cdk4 and Cdk6 kinase on RB-C-terminal fusion protein was not significant compared with Cdc2 and Cdk2 (Fig. 4A).

Both Cdc2 and Cdk2 Kinase Activity to Phosphorylate RB Are Dramatically Enhanced at the Early Phase of TGF-β1-induced Apoptosis—The most studied G1 cyclin/Cdk substrate is the product of retinoblastoma tumor suppressor protein (RB). Since the kinase activities of Cdkks were transiently activated in this study, we investigated the phosphorylation status of endogenous RB in response to TGF-β1. Western blot analysis using anti-phosphospecific RB antibody demonstrated a dramatic increase in RB phosphorylation, which showed a peak at 8 h following TGF-β1 treatment (Fig. 5A), coinciding with the activation of Cdkks observed in Fig. 4. The kinases responsible for RB phosphorylation in vivo have been reported to be members of the Cdk family, including Cdk2 in association with cyclin E and A, as well as Cdk4 and Cdk6 in association with D-type cyclins (reviewed in Ref. 49). Massive RB hyperphosphorylation in response to TGF-β1 as shown in Fig. 5A may not be explained by the slight increase in Cdk4 and Cdk6 kinase activity on RB. At least seven of potential Cdk phosphorylation sites (Ser-249, -807, and -811, and Thr-252, -373, -821, and -826) have been shown to be phosphorylated in vivo (50, 51). Therefore, our data suggest that N terminus of RB is also involved in RB hyperphosphorylation, although C-terminal fragment (amino acids 791–928) of RB has been conventionally used as an exogenous substrate for RB kinase such as Cdk2 and Cdk4. To examine which Cdk(s) play a major role for RB hyperphosphorylation, immune complex kinase assay of Cdk was performed using two types of RB substrate, including a recombinant RB-N terminus fusion protein containing RB residues 181–400 and an RB-C terminus fusion protein containing RB residues 701–928 (Fig. 5B). Surprisingly, not only Cdk2 but also Cdc2 demonstrated a significant increase in RB kinase activity on both the N and C termini of RB protein, peaking at 8 h after TGF-β1 treatment. Interestingly, N-terminal RB protein phosphorylated by activated Cdc2 revealed two discrete bands with an equal intensity, whereas the RB protein phosphorylated by activated Cdk2 demonstrated a higher intensity in the upper shifted band. This result suggests that Cdc2 and Cdk2 kinase have a differential substrate specificity or preference for specific sites at RB-N terminus. In contrast, Cdk4 revealed a very trifling increase in kinase activity on RB-N terminus fusion protein. Cdk6 showed a slight activation on both RB-N terminus and RB-C terminus. These results indicate that activated Cdc2 as well as Cdk2 are responsible for a massive RB hyperphosphorylation following TGF-β1 treatment. We further examined whether the Cdk2 or Cdc2 complex binds stably to RB to facilitate RB phosphorylation in vivo. We immunoprecipitated Cdk2- and Cdc2-containing complexes, respectively, and tested for coprecipitation of RB. As shown in Fig. 5C, Cdk2- or Cdc2-specific antibody precipitated Cdk2 or Cdc2, respectively, and coprecipitated more RB at the cell extracts prepared from cells treated with TGF-β1 for 8 h. Therefore, these results suggest that the association of RB with Cdc2 or Cdk2 complex increases at the time of activation of these kinases. In conclusion, our results strongly suggest the existence of Cdc2-RB and Cdk2-RB complexes in vivo, and RB is a physiological target of inappropriately activated Cdc2 and Cdk2 by TGF-β1.

Specific Inhibitors of Cdc2 and Cdk2 Kinase Block TGF-β1-induced Apoptosis—Recently, activation of cyclin-dependent kinases, either Cdc2 or Cdk2, has been shown to correlate with the onset of apoptosis (34–40). However, the significance of activation of specific cyclin-dependent kinases in apoptosis has been controversial in several cell culture systems induced by diverse stimuli (52–54). In order to determine whether the activation of these kinases is a required signal for TGF-β1-induced apoptosis, we examined the effect of roscovitine and olomoucine (55, 56), specific inhibitors for both Cdc2 and Cdk2, on TGF-β1-induced apoptosis. FaO cells were pretreated with roscovitine or olomoucine at the various concentrations for 1 h and then treated with TGF-β1. The extent of apoptosis was assessed by the TUNEL assay at 24 h after TGF-β1 treatment (Fig. 6A). Treatment of these inhibitors blocked the DNA fragmentation of FaO cells that is normally induced by TGF-β1 in a dose-dependent manner. The death-blocking effect of roscovitine was more potent than that of olomoucine. Cells treated
with 30 μM roscovitine or 100 μM olomoucine prior to TGF-β1 treatment were almost resistant to apoptosis (Fig. 6B). Next, we investigated whether RB hyperphosphorylation in response to TGF-β1 is affected by the treatment of 30 μM roscovitine. Western blot analysis using anti-phosphospecific RB antibody demonstrated no significant changes in the RB phosphorylation levels (Fig. 6C). To confirm whether this specific inhibitor blocks TGF-β1-induced apoptosis by the inhibition of Cdc2 and Cdk2 kinase activity to phosphorylate RB, immune complex kinase assays of Cdkks were performed using the control cell extracts and the cell extracts treated with 30 μM roscovitine and TGF-β1 for 8 h (Fig. 6D). The activities of Cdc2 and Cdk2 kinase on RB induced by TGF-β1 were almost suppressed with the treatment of this inhibitor. This result confirms that increased Cdc2 and/or Cdk2 kinase activity to phosphorylate RB is required for TGF-β1-induced apoptosis.

**Overexpression of Bcl-2 Suppresses TGF-β1-induced Apoptosis by Specifically Blocking Cdc2 Kinase Activation—Overexpression of the bcl-2 gene has been shown to block the cell death caused by diverse death stimuli in many cell types (41), although its mechanism of action still remains obscure. In addition, adenovirus E1B 19K protein, another Bcl-2 family member, is presumed to prevent the cell death, although its role in cell death has been studied less intensively than the role of Bcl-2 (57, 58, 60). First, we investigated whether overexpression of Bcl-2 or E1B 19K protein can block TGF-β1-induced cell death. Stable cell lines expressing Bcl-2 or E1B 19K were generated (Fig. 7A). Most of the Bcl-2-overexpressing sublines were completely resistant to TGF-β1-induced apoptosis as estimated by the TUNEL assay, despite slight differences in the expression of bcl-2 transcripts between them, whereas TGF-β1-induced apoptosis was partially blocked in all E1B 19K protein-expressing cell lines (Fig. 7, B and C).

In order to test whether TGF-β1-induced activation of Cdc2 or Cdk2 kinase directly correlates with the occurrence of apoptosis, the changes in Cdc2 and Cdk2 kinase activities were examined using a Bcl-2-expressing FaO subline (Bcl-2/FaO) and an E1B 19K protein-expressing FaO subline (E1B 19K/FaO), respectively (Fig. 8A). Cell extracts were isolated from the two cell lines at the indicated times after TGF-β1 treatment and subjected to a Cdc2- or Cdk2-associated histone H1 kinase assay. As shown in Fig. 8A, no significant activation of Cdc2 kinase activity was observed following TGF-β1 treatment in Bcl-2/FaO cells, whereas Cdk2 kinase activity was markedly increased in these cells, similar to the increase shown in TGF-β1-treated FaO cells (Fig. 4A). In E1B 19K/FaO cells, which showed a very slow progression of apoptosis in response to TGF-β1, there was a gradual increase in Cdc2 kinase activity up to 24 h following TGF-β1 treatment. However, Cdk2 kinase activity was still significantly activated with a peak at 8 h after TGF-β1 treatment as observed in FaO cells. The similar results were obtained in Cdc2- or Cdk2-associated kinase assay on RB (data not shown). These results demonstrate that activation of Cdc2 kinase is closely linked to TGF-β1-induced apoptosis in FaO cells. On the other hand, Cdk2 kinase can be activated in response to TGF-β1 without concomitant apoptosis. Therefore, we can conclude again that activation of Cdc2 is essential for induction of apoptosis by TGF-β1 in FaO cells, even though TGF-β1 can activate both Cdc2 and Cdk2 kinases. Also these results suggest that the anti-apoptotic function of Bcl-2 and E1B 19K protein may be mediated through inhibition of Cdc2 kinase activation. Next, we investigated whether overexpression of anti-apoptotic genes affects the changes in the phosphorylation levels of endogenous RB following TGF-β1 treatment (Fig. 8B). Whereas a very slight increase in RB phosphorylation with a peak at 8 h after TGF-β1 treatment was observed in Bcl-2-overexpressing cells, phosphorylation of RB increased gradually up to 12 h in 19K protein-overexpressing cells. These results suggest again that the apoptotic occurrence may be associated with a massive hyperphosphorylation of RB.

Next, the possible action mechanism by which Bcl-2 and 19K regulates the activation of Cdc2 and Cdk2 in response to...
TGF-β1 was investigated by the expression analysis of the related cell cycle components in the stable cell lines overexpressing Bcl-2 and 19K. No significant difference in the expression pattern of Cdk2 and cyclin D1 was observed among Bcl-2-, 19K-overexpressing cells, and control cells (pcDNA3/FaO), explaining the reason that the consequent activation of Cdk2 in response to TGF-β1 is not affected by the overexpression of these anti-apoptotic gene products (Fig. 9A). Interestingly, TGF-β1 failed to induce Cdc2 mRNA and protein in Bcl-2-expressing cells. In contrast, in 19K-expressing cells, the delayed induction of Cdc2 mRNA and protein was observed (Fig. 9A and B). Therefore, these results indicate that the blocking effect of TGF-β1-induced apoptosis by Bcl-2 or 19K may be closely associated with the suppression of induction in Cdc2 mRNA and the subsequent Cdc2 kinase activity.

In conclusion, TGF-β1-induced increases in Cdc2 and Cdk2 kinase activities significantly contribute to the transient hyperphosphorylation of RB in FaO cells. The consequent inactivation of RB may promote the failure of controlled cell cycle progression, leading to irreversible cell death.

**DISCUSSION**

Apoptosis is a complex cellular response with multiple signaling pathways and regulatory proteins. We have previously reported that activation of caspase-2 may have a crucial role at the execution stage of TGF-β1-induced apoptosis in FaO rat
hepatoma cells (12). In this study, we explored the signaling pathways at the early stages of TGF-β1-induced apoptosis. The regulatory pattern of cell cycle components after TGF-β1 treatment showed the signal for a rapid cell cycle progression, including the transient transcriptional induction of cyclin A, B, D1, and Cdc2, together with hyperphosphorylation of RB. Furthermore, the progressive decrease in the G1 cell cycle population occurred before entry into apoptosis. Therefore, TGF-β1-induced apoptosis takes a separate signaling pathway from the TGF-β1-induced cell cycle arrest pathway, which is one of the most studied aspects of TGF-β function. Lack of cellular capacity to accommodate these excessive proliferating signals induced by TGF-β1 may contribute to a failure in coordinated control at the checkpoint of cell cycle transition and a resultant irreversible cell death in FaO cells.

Expression of various cyclins (30–33) and inappropriate activation of cyclin-dependent kinases (34, 35, 37–39, 61, 62) have been implicated in apoptotic triggering of several experimental systems. However, it has been a matter of controversy whether activation of cyclin-dependent kinases is a secondary event of apoptosis, depending on the systems. De Luca et al. (52) suggested that Fas-induced changes in Cdc2 and Cdk2 kinase activities are not sufficient enough to trigger apoptosis in HUT-78 cells, and some models of apoptosis have demonstrated that enhanced activation of Cdc2 kinase is not required for apoptosis to occur (53, 54). Furthermore, the inhibitor of Cdk2 has been reported to induce apoptosis in some cells (63, 64). In contrast, evidence that Cdc2 activity is required for apoptosis is provided by the observation that overexpression of a dominant negative Cdc2 mutant blocks Fas/APO-1-induced apoptosis in Jurkats cells (40), and that cells with a temperature-sensitive Cdc2 mutant are unable to undergo apoptosis in

![Fig. 7. Effect of the overexpression of Bcl-2 and 19 kDa on TGF-β1-induced apoptosis and progression of cell cycle. A, the expression of Bcl-2 or 19-kDa mRNA in the representative cells which were stably transfected with the mammalian vectors encoding bcl-2 or adenovirus E1B 19 K gene was examined by Northern blotting. Lanes 1 and 3, FaO subline transfected with pcDNA3; lane 2, FaO subline transfected with bcl-2 gene; lane 4, FaO subline transfected with E1B 19 K gene. B, the cell survivals of the representative cells overexpressing Bcl-2, E1B 19K, and the control cells (the cells stably transfected with pcDNA3) were compared after treatment with TGF-β1 for the indicated times (0, 12, and 24 h) by a phase contrast microscopy. These experiments were performed using different clones with similar results. C, progression of cell cycle in the stable cell line expressing Bcl-2 or E1B 19 K. At the indicated times of TGF-β1 treatment, cells were fixed with 70% ethanol, stained with propidium iodide, and subjected to fluorescence-activated cell sorting analysis. Percentage of G1, G2/M, and sub-G0 phase cells were calculated by deconvolution of the DNA content histograms.](image1)

![Fig. 8. Effect of overexpressed Bcl-2 or E1B 19K protein on the activity of Cdc2 and Cdk2 kinase and on RB phosphorylation following TGF-β1 treatment. A, Cdc2- and Cdk2-associated immune complex kinase assay using histone H1. The same procedure was performed as described in Fig. 4. B, changes in the phosphorylation levels of RB in Bcl-2- and 19 kDa-overexpressing cells in response to TGF-β1. Each cell line overexpressing Bcl-2 or E1B 19K was treated with TGF-β1 for the indicated times, and cell extracts were prepared for the Western blotting to detect the phosphorylated forms of RB.](image2)
response to a variety of stimuli when cultured at the restrictive temperature (38). Inhibition of cyclin B1-Cdc2 kinase activity has been proposed as one mechanism by which some transforming oncogenes protect against apoptosis (65).

Our studies on the changes in Cdk activities during TGF-β1-induced apoptosis demonstrated that all Cdk5 analyzed in our study were activated with a peak at 8 h after TGF-β1 treatment. In our study, the significant role of Cdc2 and/or Cdk2 kinase in TGF-β1-induced apoptotic signaling was demonstrated by the result that roscovitine or olomoucine, potent Cdc2 and Cdk2 inhibitors, effectively blocked TGF-β1-induced apoptosis. In particular, a direct correlation was observed between the activation of Cdc2 and the progression of apoptosis in response to TGF-β1. Not only the apoptotic occurrence induced by TGF-β1 but also the blocking of apoptosis by roscovitine or by the overexpression of anti-apoptotic gene products such as Bcl-2 and 19K were closely associated with activation of Cdc2 kinase. Therefore, these results suggest that activation of Cdc2 is critical for TGF-β1-induced apoptosis. Interestingly, Cdk2 kinase activity was activated in response to TGF-β1 regardless of apoptotic occurrence, suggesting that only the increase in this Cdk2 activity may not be sufficient to trigger apoptosis.

Overexpression of Bcl-2 family proteins protects against the cell death induced by diverse death stimuli, although their action mechanisms are still obscure (41). Previous reports have demonstrated that the inhibition of death by Bcl-2 is associated with alterations in the expression and localization of Cdk proteins or cyclin A (35, 37). In addition, the level of Bcl-2 protein in T cells has been connected with the retardation of the G1/S transition via the sustained level of p27 (66) or through dephosphorylation of RB (43). Therefore, the protective effect of Bcl-2 against cell death may be accomplished by modulating the cell cycle progression, i.e., by increasing the length of the G1 phase. In this study, we have shown that the overexpression of Bcl-2 inhibited the induction of Cdc2 mRNA in response to TGF-β1. In particular, the complete blocking of TGF-β1-induced apoptosis by Bcl-2 may be accomplished by inhibiting the inappropriate activation of Cdc2 kinase through the suppression of TGF-β1-induced Cdc2 expression.

RB has been regarded as a major effector for G1 cell cycle arrest induced by TGF-β1 in many cell types, including epithelial cells. We present the several lines of evidence that RB serves as a target of activated Cdks in TGF-β1-induced apoptosis of FaO cells. An increase in the hyperphosphorylated forms of RB following TGF-β1 treatment coincides with the activation of all Cdks. In addition, when TGF-β1-induced apoptosis is blocked, either by the pretreatment of specific inhibitors of Cdc2/Cdk2 or by the overexpression of anti-apoptotic gene products, RB hyperphosphorylation was not observed. RB hyperphosphorylation by both Cdc2 and Cdk2 markedly increased in response to TGF-β1, whereas Cdk4 and Cdk6 demonstrated only slight increases in their kinase activities on RB. Furthermore, the association of RB with Cdc2 or Cdk2 was detected in active Cdc2 or Cdk2 immune complex at the peak of their RB kinase activities.

RB contains 16 Ser/Thr-Pro motifs, which are potential Cdk phosphorylation sites (50, 51, 67). The kinases responsible for RB phosphorylation in vivo are known to include members of the Cdk family, including Cdk2 in association with cyclin E and A as well as Cdk4 and Cdk6 in association with D-type cyclins (56). Cdc2 kinase activity to phosphorylate RB has been documented from in vitro experiments using purified Cdc2 complex (50, 68). In this study, we demonstrate the evidence for the first time that Cdc2 activated during apoptosis phosphorylates RB not only at N terminus but also at C terminus. The appearance of two discrete phosphorylated bands as shown in Cdc2 immune complex kinase assay on RB-N-terminal fusion protein (Fig. 5B) suggests that there may be more than one phosphorylation site between residues 181 and 400 of RB protein by Cdc2 kinase. Supporting our idea, Ser-289, Thr-252, and Thr-373 of RB protein have been reported to correspond closely to the consensus sequence for phosphorylation by Cdc2 kinase (50).

Evidence is emerging that various Cdks differentially phosphorylate RB at distinctive residues in vitro (68, 69). Phosphorylation at particular residues of RB may affect the binding of RB to only particular subsets of its interacting partners (70). This suggests that different arrays of RB phosphorylation by distinct Cdks may result in differential regulation of downstream effector pathways. Therefore, it will be very interesting to examine how the decision making for TGF-β1-induced apoptosis is controlled by RB hyperphosphorylation. An abrupt RB phosphorylation by activated Cdks may result in its functional loss as a coordinator at G1/S cell cycle transition and may trigger the subsequent irreversible cell death. However, we cannot exclude the possibility that the phosphorylation event at particular sites of RB by activated Cdc2 may be sufficient for apoptotic triggering through downstream signaling, such as a release of E2F.

Involvement of RB in apoptosis has already been suggested by several groups. Massive apoptotic cell death as well as inappropriate cell proliferation was observed in RB deficient mice (71). Ectopic overexpression of adenovirus E1A, human papilloma virus E7, E2F, cyclin D1, or c-Myc, all of which associate with and/or inactivate RB, has been demonstrated to be associated with cell death (72–76). RB was phosphorylated during apoptosis of Balb/c-3T3 fibroblasts induced by serum
deprivation (77). Furthermore, the underphosphorylated active form of RB plays a crucial role in protecting cells from radiation-induced apoptosis in cell line SAOS-2 (78). However, the signaling pathways leading to activation of RB kinases and the action mechanism of specific Cdk modulating RB function to trigger apoptosis need to be investigated further.

In conclusion, we demonstrated clearly that TGF-β1 induces transient up-regulation of Cdc2 and several cyclins in FaO cells. The subsequent activation of Cdc2 and Cdk2 is responsible for the marked hyperphosphorylation of RB. This RB phosphorylation event may be linked to the resultant failure in G1 cell cycle arrest, inappropriate S phase entry, and ultimately to irreversible cell death.

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