Oncogene (1997) 15, 1471–1480

Pim-1 kinase stimulates c-Myc-mediated death signaling upstream of caspase-3 (CPP32)-like protease activation

Toshihiro Mochizuki[1], Chifumi Kitanaka[1], Kohji Noguchi[1], Akinori Sugiyama[1], Shigehide Kagaya[1], Shunji Chi[1], Akio Asai[2] and Yoshiyuki Kuchino[1]

[1]Biophysics Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104; [2]Department of Neurosurgery, Tokyo University School of Medicine, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

Pim-1 oncoprotein is a serine/threonine kinase that can closely cooperate with c-Myc in lymphomagenesis, as does Bcl-2. Although the molecular mechanism of this cooperative transformation remains unknown, it is speculated that, similar to Bcl-2, Pim-1 contributes to transformation by inhibiting apoptosis. In this study, therefore, we examined the effect of Pim-1 expression on c-Myc-mediated apoptosis of Rat-1 fibroblasts triggered by serum deprivation. Our results showed that, rather than inhibiting apoptosis, Pim-1 expression stimulated c-Myc-mediated apoptosis in Rat-1 fibroblasts. Pim-1 stimulated c-Myc-mediated apoptosis through an enhancement of the c-Myc-mediated activation of caspase-3 (CPP32)-like proteases, since the suppression of this activity by a specific caspase inhibitor abolished the apoptosis stimulation by Pim-1. A kinase-defective Pim-1 mutant failed to stimulate c-Myc-mediated apoptosis, and Pim-1 expression alone in the absence of c-Myc overexpression did not induce apoptosis of serum-deprived Rat-1 cells, indicating that the kinase activity of Pim-1 and the activated c-Myc signaling pathway were required for apoptosis stimulation by Pim-1. Together, these results suggest that Pim-1 oncoprotein stimulates as a serine/threonine kinase the death signaling elicited by c-Myc at a step upstream of caspase-3-like protease activation in Rat-1 fibroblasts. Our results also suggest that Pim-1 kinase might function cooperatively with c-Myc through the phosphorylation of a factor(s) which regulates the common signaling pathway involved in c-Myc-mediated apoptosis and transformation.

Keywords: Pim-1; c-Myc; caspase; apoptosis; serine/threonine kinase; transformation

Introduction

The *pim-1* oncogene was first identified by its activation by proviral insertion in murine leukemia virus-induced T cell lymphomas (Cuypers *et al*., 1984; Selten *et al*., 1985). The oncogenic potential of *pim-1* was subsequently demonstrated in the Eμ-*pim-1* transgenic mice (van Lohuizen *et al*., 1989). Interestingly, either c-*myc* or N-*myc* was activated by proviral insertion in all T cell lymphomas that developed in the Eμ-*pim-1* transgenic mice (van Lohuizen *et al*., 1989). Conversely, pre-B cell lymphomagenesis in Eμ-*myc* transgenic mice was accelerated by the infection of Molony murine leukemia virus, whose provirus was frequently inserted near the *pim-1* gene (van Lohuizen *et al*., 1991). Moreover, double transgenic Eμ-*myc*, Eμ-*pim-1* mice exhibit pre-B cell leukemia *in utero* (Verbeek *et al*., 1991). These findings clearly indicated that *pim-1* can closely and specifically cooperate with c-*myc* in tumorigenesis, though the molecular mechanism of this cooperation remains unknown. It has been shown that *pim-1* expression rescues both lymph node cells from rapid apoptosis *in vitro* and $CD4^+/CD8^+$ double-positive thymocytes from dexamethasone-induced apoptosis *in vivo* in Eμ-*pim-1 lpr*/*lpr* mice (Möröy *et al*., 1993). By analogy to the *bcl-2* proto-oncogene, which is considered to contribute to cellular transformation by inhibiting apoptosis, it has been suggested that *pim-1* may also contribute to transformation by inhibiting apoptosis (Möröy *et al*., 1993).

The c-*myc* proto-oncogene was initially identified as the cellular homolog of the viral oncogene v-*myc*, and the critical role of c-*myc* in tumorigenesis has been well established (Henriksson and Lüscher, 1996). However, it has been also demonstrated that the deregulated expression of c-Myc accelerates or induces apoptotic cell death in cells deprived of growth factors (Askew *et al*., 1991; Evan *et al*., 1992). Thus, c-*myc* is not only a positive regulator of cell proliferation and transformation but also a positive regulator of apoptotic cell death. It is therefore expected that the inhibition of c-Myc-mediated apoptosis would enhance the oncogenic ability of c-Myc. Consistent with this idea, Bcl-2, which effectively inhibits c-Myc-mediated apoptosis (Fanidi *et al*., 1992; Bissonnette *et al*., 1992; Wagner *et al*., 1993), cooperates with c-Myc in the malignant transformation of lymphoid cells in transgenic mice (Strasser *et al*., 1990).

To gain insights into the mechanism of cooperation between *pim-1* and c-*myc*, we investigated the role of Pim-1 in c-Myc-mediated apoptosis. Here we show that Pim-1 acts as an activator rather than an inhibitor of c-Myc-mediated apoptosis induced in Rat-1 fibroblasts by serum deprivation. We show that Pim-1 does so through an enhancement of the c-Myc-mediated activation of caspase-3 (CPP32)-like proteases. In addition, using a kinase-defective mutant of Pim-1, we demonstrate that the kinase activity of Pim-1 is required for this enhancement of the c-Myc-mediated activation of caspase-3-like proteases causing apoptotic cell death. Our results indicate that oncoprotein Pim-1 as a serine/threonine kinase augments the death signal elicited by c-Myc at a step upstream of caspase-3-like protease activation in Rat-1 fibroblasts. Our findings also suggest that *pim-1* might cooperate with c-*myc* by

Correspondence: Y Kuchino
Received 2 April 1997; revised 30 May 1997; accepted 30 May 1997

stimulation of the intracellular signaling required for c-Myc-mediated transformation and apoptosis.

Results

Establishment of Rat-1 transfectants that stably express c-Myc, Pim-1, or both

To investigate the effect of Pim-1 expression on c-Myc-mediated apoptosis, we used the experimental system described by Evan *et al*. (1992), in which the overexpression of c-Myc causes apoptosis of serum-deprived Rat-1 fibroblasts. We first established Rat-1 clones that stably express c-Myc (Rat-1/c-Myc) by transfecting Rat-1 cells with a plasmid vector (pCEP4cmyc) carrying human c-myc cDNA connected with the cytomegalovirus (CMV) promoter. Consistent with the previous report (Evan *et al*., 1992), the Rat-1/c-Myc clones could grow normally in medium containing 10% fetal calf serum (FCS) but underwent typical apoptosis when deprived of serum growth factors. Among the established clones, a representative clone (Rat-1/c-Myc#7) was used for the subsequent assays. To examine the possible involvement of Pim-1 in c-Myc-mediated apoptosis, we transfected pcDNA3pim-1 expressing rat pim-1 cDNA under the control of CMV promoter into parental Rat-1 and Rat-1/c-Myc#7 cells to isolate Rat-1/Pim-1 and Rat-1/c-Myc/Pim-1 clones, respectively. After selection by G418, stable transfectants were obtained and examined by Northern blot analysis for pim-1 mRNA expression. In addition to the low level of endogenous pim-1 transcript, Rat-1/Pim-1#1, Rat-1/Pim-1#2, Rat-1/c-Myc/Pim-1#3, and Rat-1/c-Myc/Pim-1#4 cells expressed significantly high levels of transfected *pim-1* (Figure 1a, top panel). In contrast, Rat-1/c-Myc/Pim-1#1 and Rat-1/c-Myc/Pim-1#2 did not express a detectable level of transfected *pim-1*, despite the introduction of *pim-1* expression vector. We confirmed that Pim-1 expression does not significantly alter the level of c-*myc* expression either at the mRNA level (Figure 1a, middle panel) or at the protein level (Figure 1b). Consistent with the previous observation that coexpression of c-Myc and Pim-1 does not transform cultured cells (Henriksson and Lüscher, 1996), the Rat-1 transfectants expressing both c-Myc and Pim-1 did not form transformed foci even when passaged for longer than 1 month. This finding suggests that their expression alone may not be sufficient for full transformation, and that additional cellular backgrounds or environmental conditions may be required.

Pim-1 stimulates rather than inhibits c-Myc-mediated apoptosis

Using the established clones, we examined whether Pim-1 inhibits c-Myc-mediated apoptosis. When Rat-1/c-Myc#7 cells were deprived of serum growth factors (cultured in medium containing 0.1% FCS), many cells lost adherence and became round and shrunken within 24 h, as shown in Figure 2a. This process was accompanied by nuclear condensation and fragmentation, indicating that the cells were dying by apoptosis. When Rat-1/c-Myc/Pim-1#4 cells that overexpress both c-Myc and Pim-1 were similarly deprived of serum

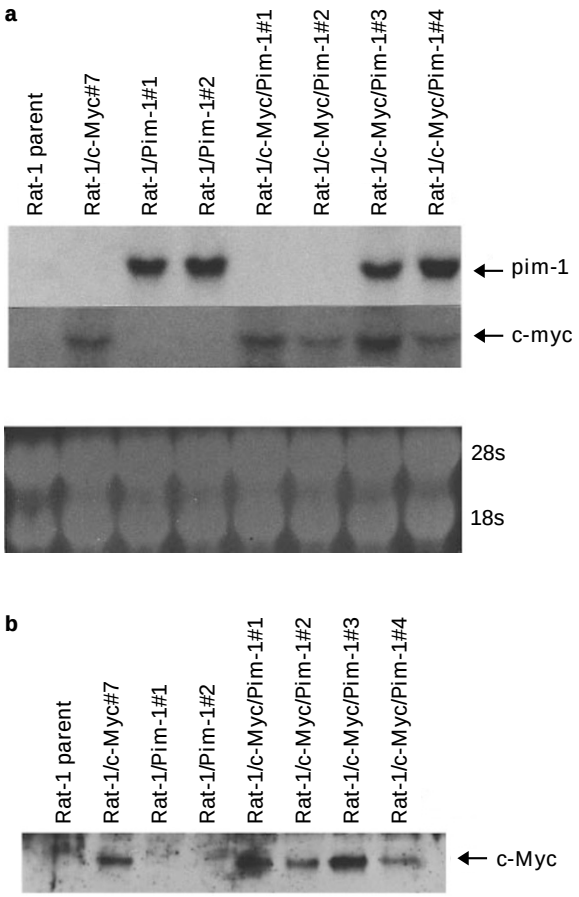


Figure 1 Characterization of Rat-1 transfectants. (**a**) Expression of the transfected pim-1 and c-myc mRNA products. Total RNAs (15 μg) were extracted from the indicated cells and subjected to Northern blot analysis using ^{32}P-labeled rat pim-1 cDNA as a probe (top panel). The same filter was deprobed and subsequently hybridized with human c-myc cDNA probe (middle panel). The bottom panel shows the ethidium bromide-stained gel to ascertain the quality and quantity of the RNA loaded. (**b**) Expression of c-Myc protein in the transfectants. Cell lysates were prepared from the indicated cells, and 40 μg of the cell lysates were subjected to Western blot analysis using anti-c-Myc polyclonal antibody

growth factors, surprisingly, a significantly larger number of dying cells was produced compared to the Rat-1/c-Myc#7 cells (Figure 2a). The cell death of Rat-1/c-Myc/Pim-1#4 cells was also accompanied by apoptotic phenotypes as found in Rat-1/c-Myc cells, including round and shrunken morphology (Figure 2a), chromatin condensation and/or nuclear fragmentation (Figure 2b), and positive staining by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Figure 2c). Quantitation of apoptotic cells by TUNEL assay revealed that, when deprived of serum, Rat-1/c-Myc/Pim-1#4 cells produced nearly twice as many TUNEL-positive cells as Rat-1/c-Myc#7 cells (Figure 2d). These results suggested that the expression of Pim-1 stimulates c-Myc-mediated apoptosis rather than inhibiting it. To confirm that the stimulation of apoptosis induction is dependent on Pim-1 expression, we analysed other Rat-1/c-Myc/Pim-1 clones. As shown in Figure 2e, when deprived of serum, the Rat-1/c-Myc/Pim-1 clones expressing Pim-1 (#3, #4) produced approximately twice as many apoptotic cells as did Rat-1/c-Myc#7, whereas Rat-1/c-Myc/Pim-1 clones not expressing Pim-

1 (#1,#2) produced numbers of apoptotic cells similar to that produced by Rat-1/c-Myc#7. Taking into account that these Rat-1/c-Myc/Pim-1 clones express comparable levels of c-Myc (Figure 1b), this result implies that the stimulation of apoptosis is dependent on Pim-1 expression and not due to clonal variation. Given that Pim-1 stimulates c-Myc-mediated apoptosis, we next asked whether the expression of Pim-1 alone can mediate the apoptosis of Rat-1 cells triggered by serum deprivation. When Rat-1/Pim-1 clones (#1,#2) were deprived of serum, very few apoptotic cells were produced as with parental Rat-1 cells (Figure 2e). Because these Rat-1/Pim-1 clones express levels of pim-1 mRNA similar to those of Rat-1/c-Myc/Pim-1#3 and #4 (Figure 1a), this result indicates that Pim-1 by itself is not able to induce apoptosis in serum-deprived Rat-1 cells. Taken together, these results suggest that although Pim-1 by itself cannot cause apoptosis under low serum culture conditions, it stimulates c-Myc-mediated apoptosis in serum-deprived Rat-1 fibroblasts.

Serine/threonine kinase activity of Pim-1 is essential for stimulating c-Myc-mediated apoptosis

It has been shown that Pim-1 is a protein-serine/threonine kinase (Telerman *et al*., 1988; Hoover *et al*., 1991; Padma and Nagarajan, 1991; Saris *et al*., 1991). We therefore investigated whether the serine/threonine kinase activity of Pim-1 is required for the stimulation of c-Myc-mediated apoptosis. For this purpose, we created a kinase-defective Pim-1 mutant (Pim-1K67M) by the conversion of Met at position 67 of the protein into Lys by site-directed mutagenesis. This Pim-1K67M mutant has been shown to lose its intrinsic kinase activity in *in vitro* kinase assays (Saris *et al*., 1991; Friedmann *et al*., 1992). We transfected pcDNA3pim-1K67M expressing this Pim-1 mutant into Rat-1/c-Myc#7 cells and isolated stable transfectants (Rat-1/c-Myc/Pim-1K67M). Among the transfectants, Rat-1/c-Myc/Pim-1K67M#7 and #16 expressed levels of exogenous pim-1 mRNA comparable to that of Rat-1/c-Myc/Pim-1#4 (Figure 3a). When Rat-1/c-Myc/Pim-1K67M#7 and #16 were cultured in medium containing 0.1% FCS, these clones produced fewer apoptotic cells than did the Rat-1/c-Myc/Pim-1#4 cells, and the number of apoptotic cells was comparable to or slightly smaller than that produced by Rat-1/c-Myc#7 cells (Figure 3c). Since Rat-1/c-Myc/Pim-1K67M#7 and #16 cells expressed levels of c-Myc protein similar to that expressed by the Rat-1/c-Myc#7 cells (Figure 3b), these results indicate that the serine/threonine kinase activity of Pim-1 is required for the stimulation of c-Myc-mediated apoptosis of Rat-1 cells triggered by serum deprivation and also suggest that this kinase-defective Pim-1 mutant (Pim-1K67M) may cause a dominant-negative effect on c-Myc-mediated apoptosis.

Pim-1 does not affect the expression levels of p53, $p27^{Kip1}$, and Bcl-2 family proteins

We next examined the effect of Pim-1 kinase on the expression regulation of factors that may be involved in the downstream processes of c-Myc-mediated apoptosis. It has been reported that wild-type p53 is required for c-Myc-mediated apoptosis (Hermeking and Eick, 1994; Wagner *et al*., 1994), during which p53 protein is stabilized by c-Myc expression (Hermeking and Eick, 1994). We therefore examined the expression level of p53 protein in Rat-1 cells expressing c-*myc* and/or *pim-1*. As Figure 4a (top panel) shows, the expression level of p53 was very low in both parental Rat-1 cells and in Rat-1 transfectants. In some experiments, the p53 protein level appeared slightly higher in c-*myc* transfectants (data not shown), but the p53 protein level was not altered by Pim-1 expression.

It has been reported that the expression of p27 cyclin-dependent kinase (cdk) inhibitor is under negative posttranslational regulation by c-Myc (Steiner *et al*., 1995). Since p27 has been shown to confer resistance against anticancer agents to tumor cells (Croix *et al*., 1996), it appeared likely that p27 has antiapoptotic activity similar to other cdk inhibitors such as p16 and p21 (Wang and Walsh, 1996; Poluha *et al*., 1996). We therefore hypothesized that c-Myc may mediate apoptosis through the down-regulation of p27, and that Pim-1 might facilitate this process. To test this idea, we examined the expression level of p27 protein in the Rat-1 transfectants. Although serum deprivation slightly increased the p27 expression, it was not significantly altered by Pim-1 expression or even by c-Myc (Figure 4a, middle panel).

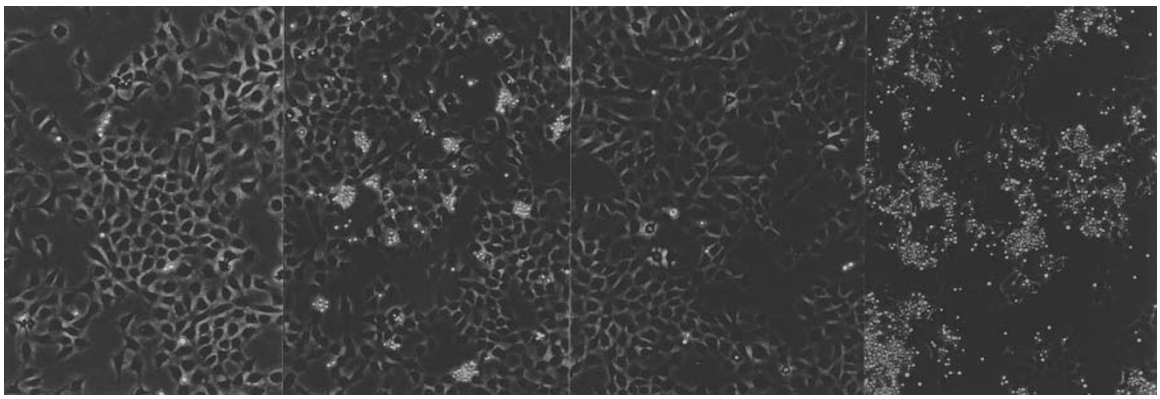
We then turned to Bcl-2 family proteins, which have been shown to positively or negatively regulate apoptosis triggered by various stimuli (Reed, 1994). We examined the expression level of the Bcl-2 family proteins in Rat-1 cells undergoing c-Myc-mediated apoptosis and the effect of Pim-1 on their expression. As shown in Figure 4b, Pim-1 expression did not affect the expression levels of the Bcl-2 family proteins Bcl-2 (Tsujimoto *et al*., 1985), Bax (Oltvai *et al*., 1993), Bcl-x_L (Boise *et al*., 1993), and Bak (Farrow *et al*., 1995; Chittenden *et al*., 1995; Kiefer *et al*., 1995). In addition, their expression levels did not change remarkably before and after apoptosis induction by serum deprivation.

Pim-1 stimulates c-Myc-mediated apoptosis through the enhancement of the c-Myc-mediated activation of caspase-3-like proteases

We recently found that caspase-3-like proteases are activated and are required for transduction of the death signal in Myc-mediated apoptosis under low serum culture conditions (SK *et al*., submitted for publication) as well as in Fas- and tumor necrosis factor (TNF)-mediated apoptosis (Tewari *et al*., 1995; Enari *et al*., 1996). We therefore investigated whether the stimulation of c-Myc-mediated apoptosis by Pim-1 depends on activation of caspase-3-like proteases. For this purpose, we first examined the caspase-3-like protease activity in parental Rat-1, Rat-1/Pim-1#1, Rat-1/c-Myc#7, Rat-1/c-Myc/Pim-1K67M#16, and Rat-1/c-Myc/Pim-1#4 cells before and after apoptosis induction (Figure 5a). Consistent with our previous observation, caspase-3-like protease activity was significantly induced in Rat-1/c-Myc#7 cells compared to Rat-1 parental cells 24 h after serum deprivation. Compared to the Rat-1/c-Myc#7 cells, the caspase-3-like protease activity induced in the Rat-

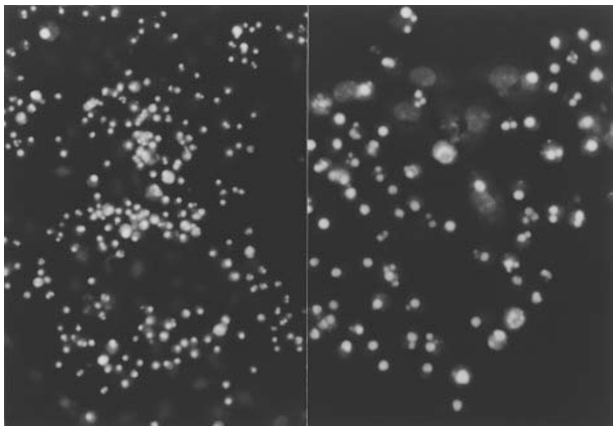
1/c-Myc/Pim-1#4 cells 24 h after serum deprivation was approximately twofold higher, and the caspase-3-like protease activity in the Rat-1/c-Myc/Pim-1K67M#16 cells was comparable to or slightly lower than that in the Rat-1/c-Myc#7 cells. The expression of Pim-1 alone failed to enhance the activation of caspase-3-like proteases upon serum deprivation, in accord with its inability to induce apoptosis by itself. Thus, the caspase-3-like protease activity correlated well with the amounts of cell death induced in these clones (compare Figures 2e and 3c with Figure 5a). These results suggested that Pim-1 kinase stimulates c-Myc-mediated apoptosis through the enhancement of c-Myc-mediated activation of caspase-3-like proteases. If this is the case, then the inhibition of caspase-3-like protease activity should also inhibit the stimulation of c-Myc-mediated apoptosis by Pim-1. To test this idea, we treated Rat-1/c-Myc#7 and Rat-1/c-Myc/Pim-1#4 cells with a specific caspase inhibitor Z-Asp-CH_2-DCB (Dolle *et al*., 1994; Mashima *et al*., 1995). In the presence of 25 μg/ml of Z-Asp-CH_2-DCB, the induction of caspase-3-like protease activity by serum deprivation was completely inhibited in both Rat-1/c-Myc#7 and Rat-1/c-Myc/Pim-1#4 cells (Figure 5b). Under this concentration of Z-Asp-CH_2-DCB, apoptosis induction was effectively inhibited to a similar level in both transfectants (Figure 5c and d). These results indicate that the enhancement of c-Myc-mediated apoptosis by Pim-1 is dependent on caspase-3-like protease activity, and the results strongly support the idea that Pim-1 oncoprotein stimulates c-Myc-mediated apoptosis in Rat-1 fibroblasts through an enhancement of the c-Myc-mediated activation of caspase-3-like proteases.

a



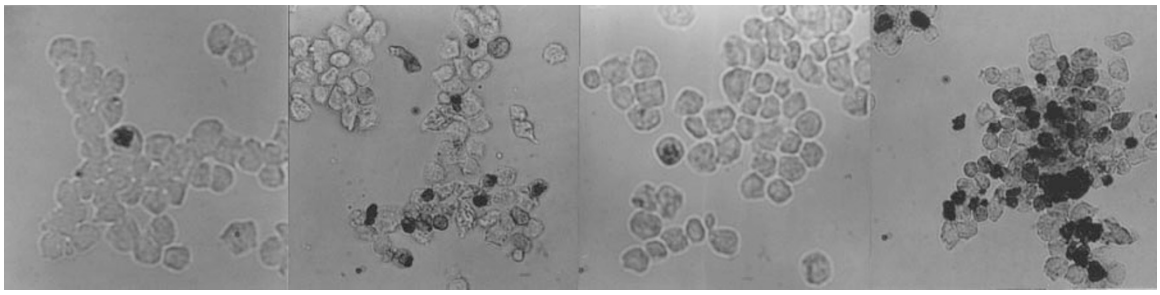
Rat-1parent | Rat-1/c-Myc#7 | Rat-1/pim-1#1 | Rat-1/c-Myc/Pim-1#4

b



Rat-1/c-Myc/Pim-1#4

c



Rat-1parent | Rat-1/c-Myc#7 | Rat-1/Pim-1#1 | Rat-1/c-Myc/Pim-1#4

Discussion

In a variety of cancers, the apoptotic signaling pathway is frequently suppressed by the expression of survival factors and/or by the inactivation of death-promoting factors, indicating that the disruption of apoptotic signaling plays an important role in carcinogenesis. In the present study, we therefore examined whether the oncoprotein Pim-1, which cooperates with c-Myc in oncogenesis, suppresses c-Myc-mediated apoptosis as does Bcl-2. However, our results indicated that Pim-1 acts as an activator rather than an inhibitor of c-Myc-mediated Rat-1 cell death triggered by serum deprivation. Our results also indicated that Pim-1 stimulates the intracellular signaling elicited by c-Myc at a step upstream of caspase-3-like protease activation. These findings appear to suggest another possible mechanism for cooperative transformation by c-Myc and Pim-1 distinct from the mechanism operating in cotransfor-

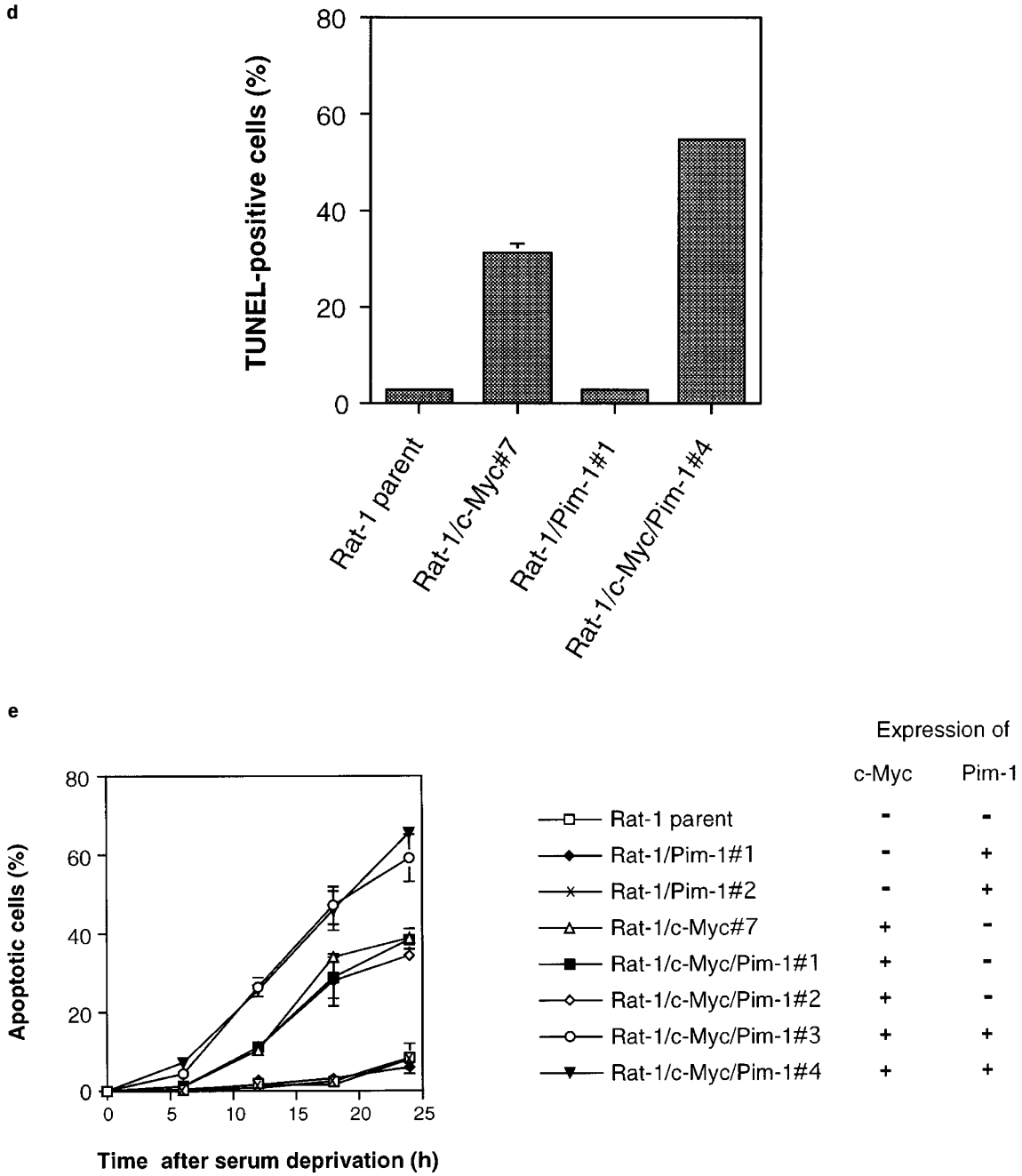


Figure 2 Expression of Pim-1 stimulates the c-Myc-mediated apoptosis. (**a**) The indicated cells were deprived of serum (0.1% FCS) for 24 h and then photographed under a phase-contrast microscope. (**b**) Rat-1/c-Myc/Pim-1#4 cells were deprived of serum for 24 h and stained with acridine orange to show chromatin condensation and/or nuclear fragmentation. Magnifications: Left, ×220, Right ×440. (**c**) The indicated cells were deprived of serum for 24 h and then subjected to TUNEL assay as described in Materials and methods. Representative fields were photographed under a phase-contrast microscope. Magnification: ×400. (**d**) Quantitation of apoptosis by TUNEL assay. Cells were treated as in (**c**), and the percentage of TUNEL-positive cells were determined. Data represent the means and standard deviation of two separate experiments. (**e**) The time course of apoptosis induction. The percentage of apoptotic cells was determined as described in Materials and methods at the indicated hours after serum deprivation. Each data point represents the mean and standard deviation of triplicate determinations

mation by c-Myc and Bcl-2. A previous study showed that the regions required for c-Myc-mediated apoptosis and transformation are tightly coupled (Evan *et al*., 1992). In addition, both c-Myc-mediated apoptosis and transformation require dimerization with Max (Amati *et al*., 1993a,b). These lines of evidence suggest that the signals elicited by c-Myc leading to apoptosis and transformation follow a common pathway at least initially and then diverge. Therefore, Pim-1 kinase may possibly interact with and stimulate the common signaling pathway for c-Myc-mediated apoptosis and transformation. Based on this idea, one could clearly understand why Pim-1 cooperates with c-Myc both in apoptosis and transformation. Moreover, it provides a better explanation for the recent observation that Bcl-2 cooperates with Pim-1 in lymphomagenesis in transgenic mice (Acton *et al*., 1992). If one assumes that Pim-1 contributes to lymphomagenesis solely by inhibiting apoptosis, it would be rather difficult to understand why Pim-1 should so efficiently cooperate with an antiapoptotic protein Bcl-2 to transform cells. In addition, Zörnig *et al*. (1996) reported that another cooperating molecule such as Gfi-1 is required for lymphomagenesis in *myc*/*pim*-1 double transgenic mice. Gfi-1 is a zinc finger protein that can repress the transcription of the *bax* gene and has been shown to inhibit cell death of thymocytes (Zörnig *et al*., 1996; Grimes *et al*., 1996). This cooperation would be well understood if one supposes that Pim-1 stimulates Myc-mediated signaling common to transformation and apoptosis and that Gfi-1 selectively suppresses the signaling pathway leading to apoptosis. Here, it should be noted that c-Myc and Pim-1 is not sufficient for lymphomagenesis and that another cooperating molecule represented by Gfi-1 is required (Zörnig *et al*., 1996). This is consistent with a previous observation and our results that coexpression of c-Myc and Pim-1 does not transform cultured cell lines

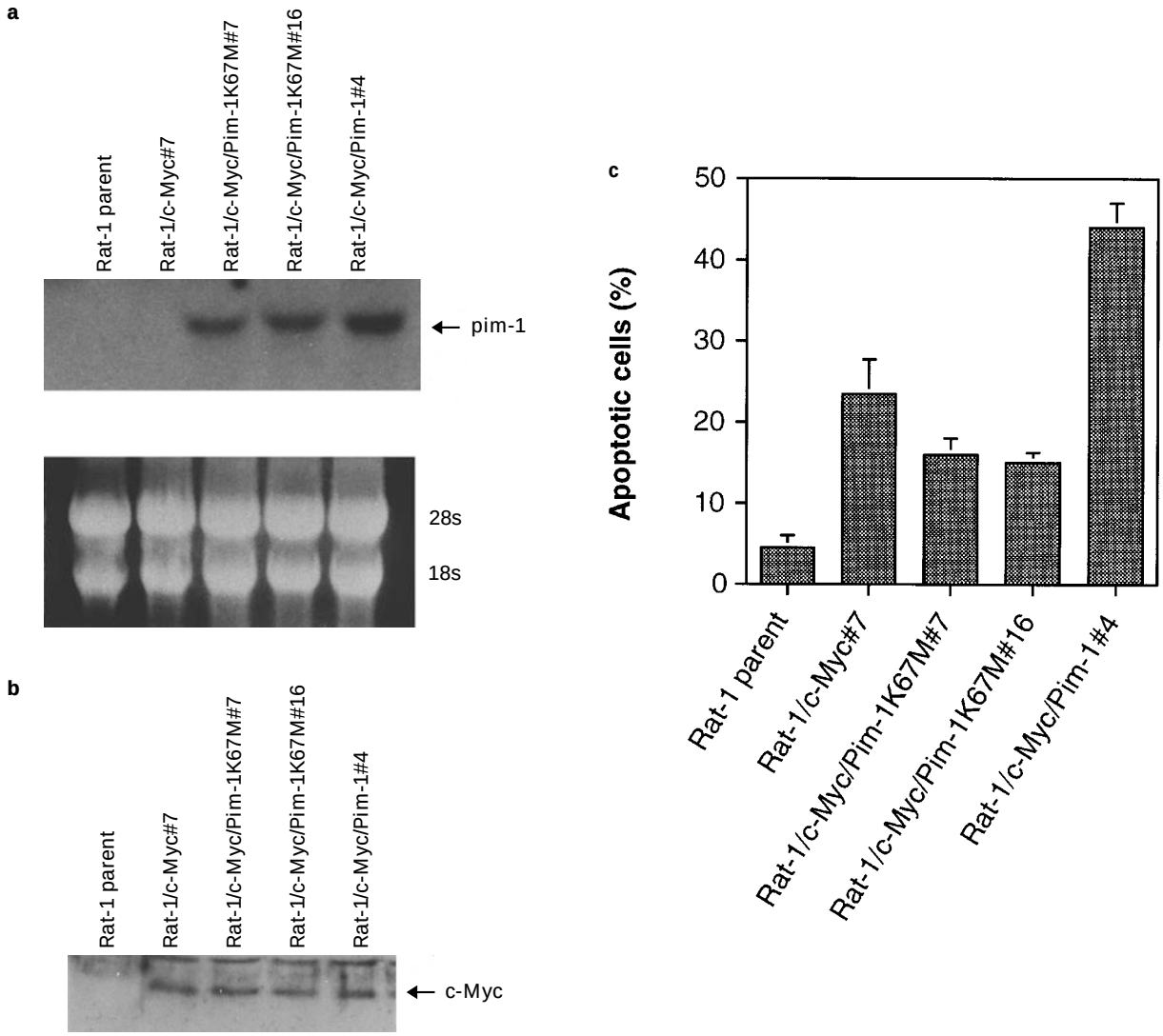


Figure 3 The serine/threonine kinase activity of Pim-1 is required for the stimulation of c-Myc-mediated apoptosis. (**a**) Expression level of pim-1K67M mRNA. Total RNAs (15 μg) were isolated from the indicated cells and subjected to Northern blot analysis using ^{32}P-labeled rat pim-1 cDNA as a probe. The ethidium bromide-stained gel is shown below to ascertain the quality and quantity of the RNA loaded. (**b**) Expression level of c-Myc protein in Rat-1/c-Myc/Pim-1K67M clones. Cell lysates (40 μg) prepared from the indicated cells were resolved on a 10% SDS-polyacrylamide gel and subjected to Western blot analysis using anti-c-Myc polyclonal antibody. (**c**) Effect of Pim-1K67M expression on c-Myc-mediated apoptosis. The indicated cells were deprived of serum (0.1% FCS) for 18 h, and the percentage of apoptotic cells was determined as described in Materials and methods. Data represent the means and standard deviation of quadruplicate determinations

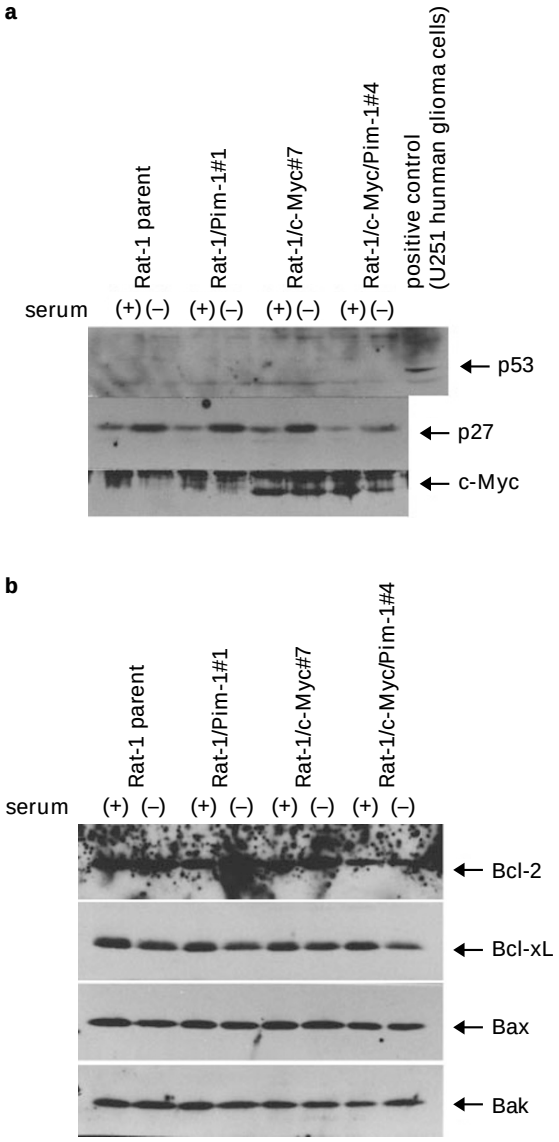


Figure 4 Expression levels of p53, $p27^{Kip1}$, and Bcl-2-related proteins are not affected by Pim-1. Cell lysates were prepared from the indicated cells which were cultured in medium containing 10% FCS [serum (+)] or 0.1% FCS [serum (−)] for 24 h, and the cell lysates (40 μg) were subjected to Western blot analysis. Antibodies for p53, $p27^{Kip-1}$, and c-Myc were used in (**a**) and antibodies for Bcl-2, Bcl-x, Bax, and Bak were used in (**b**). A cell lysate prepared from U251 human glioma (40 μg) was loaded to serve as a positive control for p53 protein

including Rat-1 fibroblasts (Henriksson and Lüscher, 1996; this study), which indicates that coexpression of c-Myc and Pim-1 is not sufficient to fully transform these cells. Although the suggestion by Möröy *et al.* (1993) that Pim-1 contributes to lymphomagenesis by apoptosis inhibition and our idea may appear somewhat contradictory, it should be emphasized that they are not mutually exclusive. It is quite possible that Pim-1 contributes to lymphomagenesis by stimulating the c-Myc-mediated signaling and by inhibiting lymphocyte apoptosis through a distinct signaling pathway at the same time. To elucidate the exact mechanism of cooperation between c-Myc and Pim-1 in lymphomagenesis, it would be important to investigate whether Pim-1 stimulates c-Myc-mediated signaling in lymphocytes, and if so, how. It would be also of interest to examine whether Pim-1 cooperates with other oncoproteins in cellular transformation and apoptosis or this cooperative effect of Pim-1 is specific to c-Myc.

To understand the mechanism by which Pim-1 modulates c-Myc-mediated apoptosis, we examined in this study the expression level of p53 tumor suppressor protein, p27 cdk inhibitor, and Bcl-2 family proteins that might be implicated in c-Myc-mediated apoptosis. However, none of these were under the control of Pim-1, although we cannot exclude the possibility that Pim-1 regulates their activity without altering their expression levels. We therefore examined the effect of Pim-1 expression on the activity of caspase-3-like proteases which we have recently found to play an essential role in c-Myc-mediated apoptosis (SK *et al.*, submitted for publication). The results indicated that the stimulation of c-Myc-mediated apoptosis by Pim-1 was dependent on the ability of Pim-1 to regulate the caspase-3-like protease activity, suggesting that Pim-1 functions upstream of caspase-3-like protease activation in the signaling pathway for c-Myc.

The result that a kinase-defective Pim-1 mutant did not stimulate c-Myc-mediated apoptosis indicates that Pim-1 stimulates c-Myc-mediated apoptosis by phosphorylation of its target molecule. Interestingly, the expression of Pim-1 *per se* induced neither caspase-3-like protease activity nor apoptosis in serum-deprived Rat-1 cells. These findings suggest that the target molecule(s) of Pim-1 kinase is absent or inaccessible to Pim-1 in the absence of c-Myc overexpression. Under c-Myc overexpression, such a molecule(s) may be abundant or accessible to Pim-1 and becomes activated after phosphorylation by Pim-1. Then, what is the target molecule of Pim-1 kinase that can modulate c-Myc-mediated apoptosis? Since c-Myc contains several sites that can be phosphorylated by a number of kinases in the transcriptional activation domain (Henriksson and Lüscher, 1996), one may speculate that c-Myc itself is the target of Pim-1. However, the possibility that Pim-1 directly phosphorylates c-Myc and stimulates its activity as a transcriptional activator is unlikely based on the following lines of evidence. First, c-Myc does not contain the substrate recognition sequence for Pim-1 kinase, $(Arg/Lys)_3$-X-Ser/Thr-X′ (X′ is likely neither a basic nor a large hydrophobic residue) (Friedmann *et al.*, 1992). Indeed, according to Saris *et al.* (1991), c-Myc protein was not phosphorylated by Pim-1 *in vitro*. Second, the coexpression of Pim-1 did not enhance the transcriptional activation of a reporter plasmid by c-Myc in transient chloramphenicol acetyl-transferase assays (TM, unpublished data). As another possibility, the transcriptional targets of c-Myc might be regulated by Pim-1 kinase, since c-Myc is considered to induce apoptosis by activating the transcription of its target genes (Henriksson and Lüscher, 1996). To date, *ECA39* (Benvenisty *et al.*, 1992), ornithine decarboxylase (*ODC*) (Bello-Fernandez *et al.*, 1993), *p53* (Reisman *et al.*, 1993), α-prothymosin (Gaubatz *et al.*, 1994), *cad* (Miltenberger *et al.*, 1995), and *Cdc25A* (Galaktionov *et al.*, 1996) have been shown to be the genes transcriptionally activated by c-Myc. Among them, Cdc25A cell-cycle phosphatase has only recently been recognized as a candidate mediator molecule of c-Myc-mediated apoptosis. Since Cdc25A has transforming activity as well (Galaktionov *et al.*, 1995a), Cdc25A may be one of the key molecules that transduce the signal

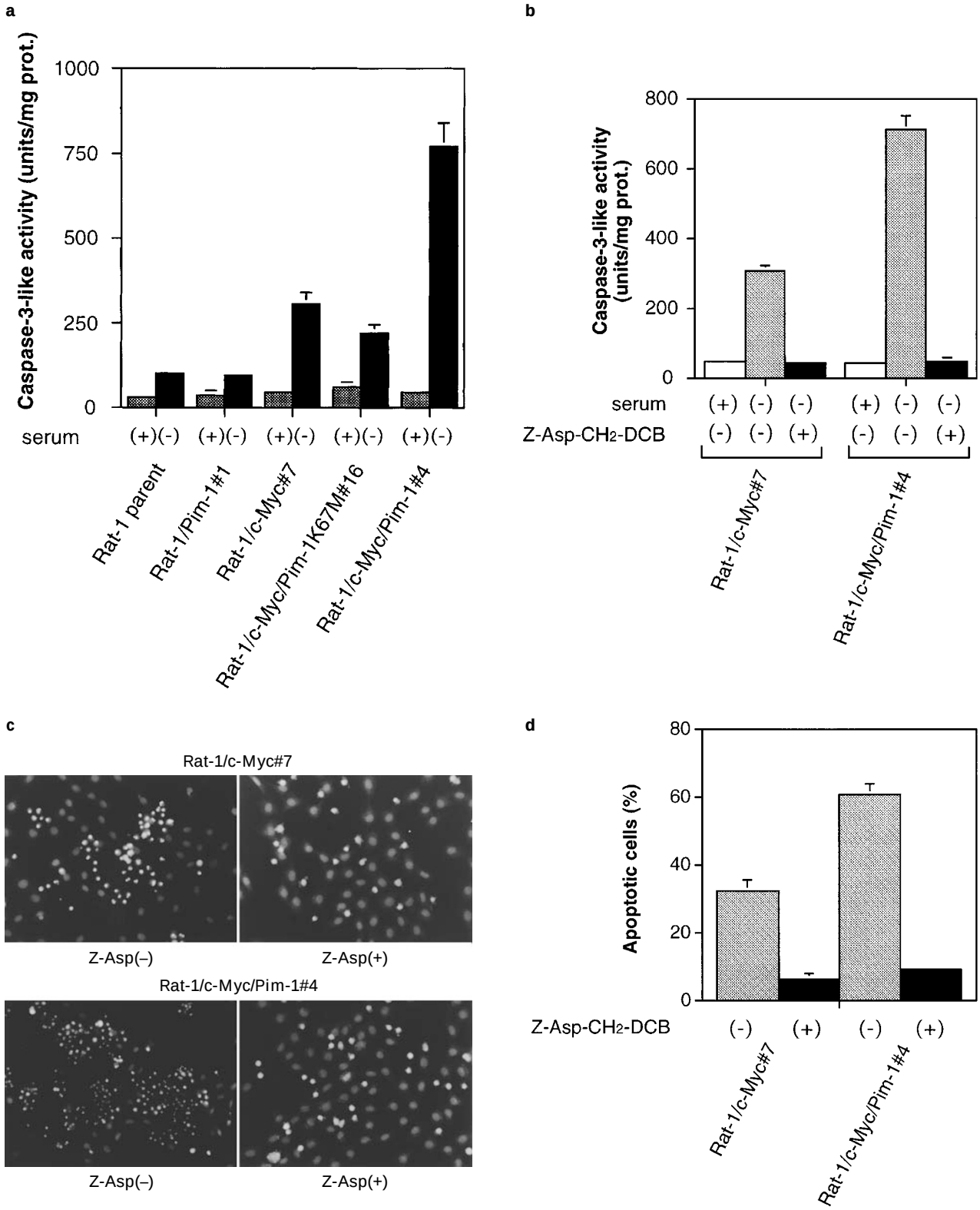


Figure 5 Pim-1 stimulates c-Myc-mediated apoptosis by enhancing the c-Myc-mediated activation of caspase-3-like proteases. (**a**) Pim-1 stimulates the c-Myc-mediated activation of caspase-3-like proteases. Cells were lysed in the assay buffer before and 24 h after serum deprivation (0.1% FCS) and were assayed for caspase-3-like activity using Ac-DEVD-MCA as a substrate. The graph presents the means and standard deviation of three separate experiments. (**b**) *In vivo* inhibition of caspase-3-like protease activity by a specific caspase inhibitor Z-Asp-CH_2-DCB. The indicated cells were cultured in medium containing 10% FCS [serum (+)] or in 0.1% FCS [serum (−)] in the presence (25 μg/ml) or absence of Z-Asp-CH_2-DCB for 24 h. Then the cell lysates were prepared and assayed for caspase-3-like protease activity. The graph presents the means and standard deviation of three separate experiments. (**c**), (**d**) Z-Asp-CH_2-DCB inhibits the stimulation of c-Myc-mediated apoptosis by Pim-1 as well as c-Myc-mediated apoptosis itself. (**c**) Rat-1/c-Myc#7 and Rat-1/c-Myc/Pim-1#4 cells were serum-deprived for 24 h in the presence (25 μg/ml) or absence of Z-Asp-CH_2-DCB. The cells were stained with acridine orange and photographed under a phase-contrast microscope. (**d**) A quantitative summary of (**c**). The indicated cells were cultured as in (**c**), and the percentage of apoptotic cells were determined 24 h after serum deprivation as described in Materials and methods. The graph represents the means and standard deviation of triplicate determinations

from c-Myc to both apoptosis and transformation. Interestingly, Cdc25A contains a peptide sequence that can be phosphorylated by Pim-1 kinase (TM, unpublished observation). It would therefore be possible that Pim-1 stimulates c-Myc-mediated apoptosis through the phosphorylation and activation of the phosphatase activity of Cdc25A, similar to another serine/threonine kinase, Raf-1, which has been shown to bind and activate the phosphatase activity of Cdc25A (Galaktionov *et al*., 1995b). We are now performing experiments to test this idea. Whether or not Cdc25A is a substrate of Pim-1 kinase, the identification of the phosphorylation targets of Pim-1 will provide important information in elucidating the signaling pathway involved in c-Myc-mediated apoptosis and transformation.

Materials and methods

Cell culture

Unless otherwise indicated, Rat-1 cells and their derivative clones were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui) supplemented with 10% FCS (Immuno-Biological Laboratories), 100 U/ml of penicillin G and 100 μg/ml of streptomycin (Life Technologies), and 0.6 mg/ml glutamine (Nissui), at 37°C in 5% CO_2. Stable Rat-1 transfectants were maintained in the presence of appropriate combinations of the selection drugs G418 (200 μg/ml, Life Technologies) and hygromycin B (200 U/ml, Calbiochem).

Plasmids and transfection

To amplify rat pim-1 cDNA, a set of oligodeoxynucleotide primers were synthesized based on the rat cDNA sequence reported previously by Wingett *et al*. (1992): forward primer 5′-TCGAATTCGATGCTCTTGTCCAAGATC-3′ and reverse primer 5′-TCGAATTCTGTCCCTGCATCGTAGAGC-3′, each of which contained a restriction enzyme site (*Eco*RI) at their 5′ termini to facilitate the subcloning of the PCR product. The pim-1 cDNA amplified by this primer set is expected to encode only the 34 kDa Pim-1 protein (Saris *et al*., 1991). Poly(A)$^+$ RNA (1 μg) from rat thymus was subjected to reverse transcription, and the product was amplified by PCR using the above primers. The amplified rat pim-1 cDNA was subcloned into the *Eco*RI site of the expression vector pcDNA3 (Invitrogen) to construct pcDNA3pim-1. pcDNA3pim-1K67M was constructed by using the Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. For this mutagenesis, pcDNA3pim-1 was used as a template, and synthetic oligodeoxynucleotides 5′-GCCGGTGGCCATCATGCACGTGGAGAAGG-3′ and 5′-CCTTCTCCACGTGCATGATGGCCACCGGC-3′ were used as sense and antisense primers, respectively. The plasmid pCEP4cmyc, which drives the expression of human c-myc cDNA, has been described (Asai *et al*., 1994). Rat-1/c-Myc cells were established by transfecting pCEP4cmyc into Rat-1 cells by the lipofectin-mediated transfection method described previously (Kitanaka *et al*., 1995) and selection against hygromycin B (400 U/ml). The plasmids pcDNA3pim-1 and pcDNA3pim-1K67M were transfected into Rat-1 cells or Rat-1/c-Myc#7 cells, and resistant clones were selected against G418 (400 μg/ml).

Northern blotting

Total RNA was extracted from exponentially growing cells using the ISOGEN reagent according to the manufacturer's protocol (Nippon Gene). The RNA samples (15 μg/lane) were separated in a 1.9% formaldehyde-1.0% agarose gel and transferred to a nylon membrane. Rat pim-1 and human c-myc cDNAs were labeled with ^{32}P-dCTP using the Oligolabeling kit (Pharmacia) according to the manufacturer's protocol. The hybridization and washing conditions were as described (Kitanaka *et al*., 1995).

Western blotting

Cells (7×10^5) were seeded into 100 mm dishes and transferred 24 h later to medium containing either 10% or 0.1% FCS. After being cultured for another 24 h, the cells were lysed in 0.1 ml of lysis buffer (25 mM Tris-HCl [pH 7.6], 50 mM NaCl, 2% NP-40, 0.5% sodium deoxycholate, 0.2% SDS). After centrifugation, the supernatants were collected as protein samples. Protein samples (40 μg protein/lane) were loaded and separated by electrophoresis in an SDS-polyacrylamide gel. Proteins were electrically transferred to a nitrocellulose membrane. The membrane was probed with the appropriate primary antibodies (N-262 for c-Myc, N-19 for Bcl-2, N-20 for Bax, G-23 for Bak, S-18 for Bcl-x, C-19 for p27 [Santa Cruz] and Ab-1 for p53 [Oncogene Science]) in antibody buffer (PBS containing 5% non-fat milk and 0.1% Tween 20) for 2 h at room temperature. After washing with antibody buffer, the membrane was subsequently incubated with HRP-Rat Anti-Rabbit IgG or HRP-Goat Anti-Mouse IgG (ZYMED) as a secondary antibody for 1 h at room temperature. The membrane was then washed with antibody buffer lacking non-fat milk, and bound antibodies were detected by an ECL Western blotting detection reagent (Amersham).

Cell death assays

For serum deprivation, cells (5.0×10^5) were seeded into 100 mm dishes and transferred 24 h later to medium containing 0.1% FCS. Apoptotic cells were identified by their loss of adherence, and apoptotic change of the nuclei was confirmed by staining the cells with 0.5 μg/ml of acridine orange (Sigma). Apoptosis was also confirmed by TUNEL assay using *in situ* Apoptosis Detection Kit (TaKaRa) according to the manufacturer's instructions. Briefly, collected cells were fixed in PBS containing 4% formaldehyde and then blocked with methanol containing 0.3% hydrogen peroxide. After permeabilisation, cells were incubated with terminal deoxynucleotidyl transferase in the presence of FITC-labeled dUTP. Incorporated dUTPs in the apoptotic cells were detected by incubation with an HRP-conjugated anti-FITC antibody and visualized by Liquid DAB-Plus Substrate Kit (ZYMED) according to the manufacturer's instructions. The percentage of apoptotic cells was determined as $100 \times$(the number of apoptotic cells relative to the total cell count). To examine the inhibition of apoptosis by Z-Asp-CH_2-DCB (Peptide Institute), cells (3×10^5) were seeded into 60 mm dishes and transferred 24 h later to medium containing 0.1% FCS and 25 μg/ml Z-Asp-CH_2-DCB.

Measurement of protease activity

Total cells (both adherent and detached) were collected and lysed with lysis buffer (10 mM HEPES-KOH [pH 7.4], 2 mM EDTA, 0.1% CHAPS, 1 mM PMSF, 5 mM DTT) for 20 min on ice. After centrifugation, the supernatants were collected as lysates. For the measurement of protease activity, 10 μg of lysates diluted to 20 μl with lysis buffer were mixed with 20 μl of $2 \times$ ICE buffer (40 mM HEPES-KOH [pH 7.4], 20% [v/v] glycerol, 1 mM PMSF, 4 mM DTT) containing 40 μM tetrapeptide substrate acetyl Asp-

Glu-Val-Asp α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) (Peptide Institute) and incubated at 37°C for 1 h. After the addition of 1 ml of distilled water, the fluorescence of the reaction mixture was determined using a spectrofluorometer. The excitation and emission wavelengths were 380 nm and 460 nm, respectively. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol 7-amino-4-methyl-coumarin (AMC) per min at 37°C.

Acknowledgements
This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the 2nd-term Comprehensive 10-Year Strategy for Cancer Control and by grants from the Ministry of Health and Welfare of Japan and from the Ministry of Education, Science and Culture of Japan for cancer research to YK. TM is a recipient of Research Resident Fellowship from the Foundation for the Promotion of Cancer Research.

References

Acton D, Domen J, Jacobs H, Vlaar M, Korsmeyer S and Berns A. (1992). *Collaboration of PIM-1 and Bcl-2 in lymphomagenesis: Mechanisms in B-Cell Neoplasia*. Peter M and Melchers M (eds). Springer-Verlag: Berlin, pp. 293–298.

Amati B, Brooks MW, Levy N, Littlewood TD, Evan GI and Land H. (1993a). *Cell*, **72,** 233–245.

Amati B, Littlewood TD, Evan GI and Land H. (1993b). *EMBO J.*, **12,** 5083–5087.

Asai A, Miyagi Y, Hashimoto H, Lee SH, Mishima K, Sugiyama A, Tanaka H, Mochizuki T, Yasuda T and Kuchino Y. (1994). *Cell Growth Differ.*, **5,** 1153–1158.

Askew DS, Ashmun RA, Simmons BC and Cleveland JL. (1991). *Oncogene*, **6,** 1915–1922.

Bello-Fernandez C, Packham G and Cleveland JC. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 7804–7808.

Benvenisty N, Leder A, Kuo A and Leder P. (1992). *Genes Dev.*, **6,** 2513–2523.

Bissonnette RP, Echeverri F, Mahboubi A and Green DR. (1992). *Nature*, **359,** 552–554.

Boise LH, González-García M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nuñez G and Thompson CB. (1993). *Cell*, **74,** 597–608.

Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI and Guild BC. (1995). *Nature*, **374,** 733–736.

Croix BS, Florence VA, Rak JW, Flanagan M, Bhattacharya N, Slingerland JM and Kerbel RS. (1996). *Nature Med.*, **2,** 1204–1210.

Cuypers HT, Selten G, Quint W, Zijlstra M, Maandag ER, Boelens W, van Wezenbeek P, Melief C and Berns A. (1984). *Cell*, **37,** 141–150.

Dolle RE, Hoyer D, Prasad CVC, Schmidt SJ, Helaszek CT, Miller RE and Ator MA. (1994). *J. Med. Chem.*, **37,** 563–564.

Enari M, Talanian RV, Wong WW and Nagata S. (1996). *Nature*, **380,** 723–726.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell*, **69,** 119–128.

Fanidi A, Harrington EA and Evan GI. (1992). *Nature*, **359,** 554–556.

Farrow SN, White JHM, Martinou I, Raven T, Pun K, Grinham CJ, Martinou J and Brown R. (1995). *Nature*, **374,** 731–733.

Friedmann M, Nissen MS, Hoover DS, Reeves R and Magnuson NS. (1992). *Archive Biochem. Biophys.*, **298,** 594–601.

Galaktionov K, Lee AK, Eckstein J, Draetta G, Meckler J, Loda M and Beach D. (1995a). *Science*, **269,** 1575–1577.

Galaktionov K, Jessus C and Beach D. (1995b). *Genes Dev.*, **9,** 1046–1058.

Galaktionov K, Chen X and Beach D. (1996). *Nature*, **382,** 511–517.

Gaubatz S, Meichle A and Eilers M. (1994). *Mol. Cell. Biol.*, **14,** 3853–3862.

Grimes HL, Gilks CB, Chan TO, Porter S and Tsichlis PN. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 14569–14573.

Henriksson M and Lüscher B. (1996). *Adv. Cancer Res.*, **68,** 109–182.

Hermeking H and Eick D. (1994). *Science*, **265,** 2091–2093.

Hoover D, Friedmann M, Reeves R and Magnuson NS. (1991). *J. Biol. Chem.*, **266,** 14018–14023.

Kiefer MC, Brauer MJ, Powers VC, Wu JJ, Umansky SR, Tomei LD and Barr PJ. (1995). *Nature*, **374,** 736–739.

Kitanaka C, Sugiyama A, Kanazu S, Miyagi Y, Mishima K, Asai A and Kuchino Y. (1995). *Cell Death Differ.*, **2,** 123–132.

Mashima T, Naito M, Kataoka S, Kawai H and Tsuruo T. (1995). *Biochem. Biophys. Res. Commun.*, **209,** 907–915.

Miltenberger RJ, Sukow KA and Farnham PJ. (1995). *Mol. Cell. Biol.*, **15,** 2527–2535.

Möröy T, Grzeschiczek A, Petzold S and Hartmann KU. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 10734–10738.

Oltvai ZN, Milliman CL and Korsmeyer SJ. (1993). *Cell*, **74,** 609–619.

Padma R and Nagarajan L. (1991). *Cancer Res.*, **51,** 2486–2489.

Poluha WP, Poluha DK, Chang B, Crosbie NE, Schonhoff CM, Kilpatrick DL and Ross AH. (1996). *Mol. Cell. Biol.*, **16,** 1335–1341.

Reed JC. (1994). *J. Cell Biol.*, **124,** 1–6.

Reisman D, Elkind NB, Roy B, Beamon J and Rotter V. (1993). *Cell Growth Differ.*, **4,** 57–65.

Saris CJM, Domen J and Berns A. (1991). *EMBO J.*, **10,** 655–664.

Selten G, Cuypers HT and Berns A. (1985). *EMBO J.*, **4,** 1793–1798.

Steiner P, Philipp A, Lukas J, Godden-Kent D, Pagano M, Mittnacht S, Bartek J and Eilers M. (1995). *EMBO J.*, **14,** 4814–4826.

Strasser A, Harris AW, Bath ML and Suzanne C. (1990). *Nature*, **348,** 331–333.

Telerman A, Amson R, Zakut-Houri R and Givol D. (1988). *Mol. Cell. Biol.*, **8,** 1498–1503.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM. (1995). *Cell*, **81,** 801–809.

Tsujimoto Y, Cossman J, Jaffe E and Croce CM. (1985). *Science*, **228,** 1440–1443.

van Lohuizen M, Verbeek S, Krimpenfort P, Domen J, Saris C, Radaszkiewicz T and Berns A. (1989). *Cell*, **56,** 673–682.

van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H and Berns A. (1991). *Cell*, **65,** 737–752.

Verbeek S, van Lohuizen M, van der Valk M, Domen J, Kraal G and Berns A. (1991). *Mol. Cell. Biol.*, **11,** 1176–1179.

Wagner AJ, Small MB and Hay N. (1993). *Mol. Cell. Biol.*, **13,** 2432–2440.

Wagner AJ, Kokontis JM and Hay N. (1994). *Genes Dev.*, **8,** 2817–2830.

Wang J and Walsh K. (1996). *Science*, **273,** 359–361.

Wingett D, Reeves R and Magnuson NS. (1992). *Nucleic Acids Res.*, **20,** 3183–3189.

Zörnig M, Schmidt T, Karsunky H, Grzeschiczek A and Möröy T. (1996). *Oncogene*, **12,** 1789–1801.