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Wortmannin enhances activation of CPP32 (Caspase-3) induced by TNF or anti-Fas

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Abstract

CPP32/apopain (Caspase-3), a protease of the Ced-3/ICE family, is a central mediator in the apoptosis induced by TNF or anti-Fas. In this study we demonstrate that wortmannin, an inhibitor of PI-3K, enhances the activation of CPP32 (Caspase-3) and DNA fragmentation in TNF-treated U937 cells and anti-Fas-treated Jurkat cells. Caspase-3-like activity, Ac-DEVD-MCA cleavage activity, is enhanced by wortmannin in the range of the concentration (1 – 100 nM) specifically inhibiting PI-3K. LY294002, another PI-3K inhibitor, also enhances Caspase-3-like activity, but inhibitors for myosin light chain kinase and calmodulin dependent kinase do not have any effect on the Caspase-3-like activity. Wortmannin (1 – 100 nM) enhances the processing of Caspase-3 (32K) into active form (17K) in TNF- or anti-Fas-treated cells, but not in untreated cells. These observations suggest that inhibition of PI-3K induces the activation of processing enzyme of Caspase-3 or increases the susceptibility of Caspase-3 to the processing enzyme. PI-3K seems to protect the cells from apoptosis by suppressing the activation of Caspase-3.

Keywords: apoptosis; Caspase-3 (CPP32); PI-3K; wortmannin; TNF; Fas

Abbreviations: PARP, poly ADP-ribose polymerase; PI-3K, phosphatidylinositol-3 kinase

Introduction

Apoptosis is of fundamental importance to biological processes in embryogenesis, the immune system, and the nervous system (Ellis *et al*, 1991). Considerable evidence suggests that Ced-3/ICE-cysteine proteases (Caspases) are components of the mammalian cell death pathway (Yuan *et al*, 1993; Miura *et al*, 1993). CPP32/apopain (Caspase-3), which is most closely related to the CED-3 among Caspases (Fernandes-Alnemri *et al*, 1994), is important in apoptosis induced by anti-Fas or TNF (Tewari and Dixit, 1995; Tewari *et al*, 1995); Poly ADP-ribose polymerase (PARP), an enzyme involved in DNA repair and genome surveillance and integrity, is one of a potential substrates for Caspase-3 during apoptosis (Tewari and Dixit, 1995). Inhibition of Caspase-3 activation attenuates apoptosis induced by anti-Fas or TNF (Tewari *et al*, 1995). Caspase-3 (32K) is processed into active form (17K and 12K) during apoptosis induced by anti-Fas or TNF (Nicholson *et al*, 1995; Schlegel *et al*, 1996).

Determination of the molecular mechanism for the activation of the proteases by the receptors has progressed recently. Fas and TNFR1 share a homologous region, appropriately designated the death domain, required to signal apoptosis (Itoh and Nagata, 1993; Tartaglia *et al*, 1993). Both receptors interact with a related set of signal-transducing molecules containing the death domain, TNFR1-associated death domain (TRADD), Fas-associating protein with death domain (FADD)/MORT1 and receptor-interacting protein (RIP) (Cleveland and Ihle, 1995; Hsu *et al*, 1995; Chinnaiyan *et al*, 1995; Stanger *et al*, 1995; Boldin *et al*, 1995). The dominant-negative version of FADD blocks TNF- and anti-Fas-mediated apoptosis, suggesting that FADD functions as the common signaling conduit for TNF-and anti-Fas-mediated cell death (Chinnaiyan *et al*, 1996b; Hsu *et al*, 1996). FLICE/MACH/Mch-5 (Caspase-8), a novel cysteine protease of the Ced-3/ICE family containing a prodomain homologous to FADD, directly binds with the death effector domain of FADD (Muzio *et al*, 1996; Boldin *et al*, 1996; Fernandes-Alnemri *et al*, 1996). Fas or TNFR1 utilizes the adaptor protein FADD to engage Caspase-8, the apical component of a proteolytic cascade made up of Caspases.

On the other hand, protein phosphorylation suppresses the apoptosis of various cells; activation of PKC maintains CTL cell viability in the absence of IL-2, and a tyrosine kinase-dependent signal prevents glucocorticoid-induced apoptosis (Walker *et al*, 1993). Apoptosis of hematopoietic cells is suppressed by signaling through the IL-3 and GM-CSF receptors (Kinoshita *et al*, 1995). Proteolytic cascade of Caspases is also involved in the apoptosis induced by inhibition of phosphorylation signals. For instance, staurosporine, an inhibitor for protein kinase C, induces apoptosis and activates Caspase-3 at high concentration (Bertrand *et al*, 1994; Chinnaiyan *et al*, 1996a).

PI-3K, which is associated with tyrosine kinase receptor, is activated by various growth factors such as EGF, insulin, insulin-like growth factor, PDGF and NGF (Cantley *et al*, 1991; Baxter *et al*, 1995). Wortmannin, a specific inhibitor of PI-3K (Arcaro and Wymann, 1993), inhibits the ability of NGF to prevent apoptosis of PC12 cells induced by a withdrawal of serum (Yao and Cooper, 1995). Recently, we showed that Caspase-3-like activity, cleavage of Ac-DEVD-MCA, is increased in DRG neurons by a withdrawal of

serum (Mukasa *et al*, 1997a). Moreover, wortmannin enhances Caspase-3-like activity during neuronal differentiation of P19 EC cells induced by retinoic acid (Mukasa *et al*, 1997b). Thus PI-3K seems to play a role as negative regulator for the activation of Caspase-3-like protease in the apoptosis of neuronal cells. To clarify a biological role of PI-3K in the apoptosis, we examined the effect of wortmannin on the apoptosis induced by TNF and anti-Fas.

Here we demonstrate that inhibition of PI-3K enhances the activation of Caspase-3, cleavage of Caspase-3, during apoptosis induced by TNF or anti-Fas.

Results

Wortmannin does-dependently inhibit the PI-3K in U937 cells and Jurkat cells (Figure 1A) The synthesis of phosphatidylinositol 3-phosphate (PI-3P), a product of PI-3K, was decreased by wortmannin at 1 nM and completely inhibited at more than 100 nM. Since wortmannin enhanced the cell death of TNF-treated U937 cells and anti-Fas treated Jurkat cells at 100 nM (unpublished observation), we examined the effect of wortmannin on the cleavage of PARP, a natural substrate for Caspase-3, in the TNF-treated U937 cells and anti-Fas-treated Jurkat cells. TNF induced the partial cleavage of PARP at 50 U/ml and anti-Fas did not induce the cleavage of PARP at 25 ng/ml. Wortmannin (100 nM) clearly enhanced the cleavage of PARP in both TNF (50 U/ml)-treated U937 cells and anti-Fas (25 ng/ml)-treated Jurkat cells (Figure 1B). These results suggest that inhibition of PI-3K is closely associated with the activation of Caspase-3-like protease.

To examine the effects of wortmannin on the activation of Caspase-3-like protease and apoptosis induced by TNF or anti-Fas in more detail, Caspase-3-like activity and Caspase-1-like activity were measured by using a synthetic peptide of PARP (Ac-DEVD-MCA) and a pro IL-1β (Ac-YVAD-MCA) as substrates, respectively. Caspase-3-like activity was dose- and time-dependently increased by both TNF and anti-Fas (Figure 2A). Caspase-3-like activity increased in 3 h and achieved a maximum level at more than 100 U/ml of TNF or 100 ng/ml of anti-Fas. Wortmannin enhanced Caspase-3-like activity induced by TNF (50 U/ml) or anti-Fas (25 ng/ml) in a different manner (Figure 2A). Wortmannin time-dependently enhanced Caspase-3-like activity of anti-Fas-treated Jurkat cells, while it transiently increased Caspase-3-like activity of TNF-treated U937 cells in 3 h. Caspase-3-like activity enhanced by wortmannin was 2–4-fold greater than the maximum level of Caspase-3-like activity induced by TNF (200 U/ml) or anti-Fas (200 ng/ml) alone (Figure 2B). However, wortmannin (100 nM) alone slightly increased Caspase-3-like activity in Jurkat cells in 12 h and transiently increased it in U937 cells in 7 h. Thus wortmannin and TNF or wortmannin and anti-Fas synergistically induced the Caspase-3-like activity. However, Caspase-1-like activity was not enhanced by wortmannin.

Wortmannin (1–1000 nM), which specifically inhibits PI-3K (Figure 1A), dose-dependently enhanced Caspase-3-like activity in the TNF (50 U/ml)-treated U937 cells or anti-Fas (25 ng/ml)-treated Jurkat cells (Figure 3A). Corresponding to the increase of Caspase-3-like activity, wortmannin doses-dependently enhanced DNA fragmentation induced by TNF (50 U/ml) or anti-Fas (25 ng/ml) (Figure 3B). Even at 1 nM, wortmannin enhanced Caspase-3-like activity and DNA fragmentation. At 1000 nM, wortmannin alone slightly, but significantly, has an ability to induce Caspase-3-like activity and DNA fragmentation.

Since, however, wortmannin also inhibits myosin light chain kinase at more than 1000 nM (Nakanishi *et al*, 1992), we examined whether inhibition of myosin light chain kinase or other kinases may enhance the Caspase-3-like activity in the Jurkat cells treated with anti-Fas. LY294002, another PI-3K inhibitor, also enhanced Caspase-3-like activity at the concentration (10–50 μM) inhibiting PI-3K. ML-9 (myosin light chain kinase inhibitor) as well as KN-93 (calmodulin kinase inhibitor) had no effects on the Caspase-3-like activity of anti-Fas-treated Jurkat cells at 10 μM (Figure 4). Similar results were obtained in the case of TNF-treated U937 cells except for the effect of KN-93 (unpublished observation).

Since Caspase-3 (32K) is processed into active form (17K) during apoptosis induced by anti-Fas (Nicholson *et al*, 1995; Schlegel *et al*, 1996), we examined the processing of Caspase-3 molecules during apoptosis enhanced by wortmannin (Figure 6). We prepared cleave-site directed antiserum against Caspase-3 (anti-p17). Anti-Caspase-3 (anti-p32) reacted with a band with 32K (p32), but not an active fragment with 17K (p17), while anti-p17 reacted with p17, but not p32 (Figure 5). These results show that anti-p17 specifically recognizes the cleavage site of Caspase-3 only after its cleavage.

The processing of p32 into p17, which was slightly detected in the TNF (50 U/ml)-treated U937 cells and anti-Fas (25 ng/ml)-treated Jurkat cells, was dose-dependently enhanced by wortmannin (1–100 nM). However, the processing was not detected in the cells treated with wortmannin (1–100 nM) alone (Figure 6). Wortmannin induced the processing of Caspase-3 only at high concentration, more than 1000 nM (unpublished observation).

Discussion

PI-3K phosphorylates the D-3 position of the inositol ring of phosphatidylinositol and produces PI-3P, which is distinct from the major mono-phosphate form of phosphatidylinositol, phosphatidylinositol 4-phosphate (Carpenter and Canter, 1990). Considerable evidence suggests that inhibition of PI-3K is involved in the apoptosis of neuronal cells induced by a withdrawal of serum or retinoic acid (Yao and Cooper, 1995; Mukasa *et al*, 1997b). However, it has not been shown whether PI-3K regulates the apoptosis induced by TNF or anti-Fas.

We examined the effects of wortmannin on the PI-3K activity, Caspase-3-like activity, i.e., cleavage of PARP and Ac-DEVD-MCA, and DNA fragmentation in the TNFR1- or Fas-mediated apoptosis. Wortmannin enhances Caspase-3-like activity and DNA fragmentation in the 1–1000 nM range of concentration, in which PI-3K activity is specifically

inhibited (Figures 1 and 3). These results suggest that wortmannin enhances Caspase-3-like activity and the DNA fragmentation induced by TNF or anti-Fas by inhibiting PI-3K. Since, however, wortmannin also inhibits myosin light chain kinase and PI-4K at more than 1000 nM and 100 nM, respectively (Nakanishi *et al*, 1992; Okada *et al*, 1994), we examined whether inhibition of myosin light chain kinase also enhances Caspase-3-like activity induced by anti-Fas. ML-9, a myosin light chain kinase inhibitor, had no effect on Caspase-3-like activity induced by anti-Fas (Figure 4), while LY294002, another PI-3K inhibitor, enhanced it. Thus PI-3K, but not myosin light chain kinase, must be involved in the negative regulation of the activation of Caspase-3-like protease. However, we do not exclude the possibility that PI-4K, which is inhibited by wortmannin at more than 100 nM (Okada *et al*, 1994), is also involved in the inactivation of Caspase-3-like protease, because phosphatidylinositol-3,4-bisphosphate, a product of PI-3K and PI-4K, activates serine-threonine protein kinase Akt, which inhibits the apoptosis of neuronal cells induced by serum deprivation (Dudek *et al*, 1997; Franke *et al*, 1997).

Many Caspases such as Caspase-3, Mch-3 (Caspase-7) and Caspase-8, have the ability to cleave Ac-DEVD-MCA. To examine whether the enhancement of the cleavage of Ac-DEVD-MCA is due to the activation of Caspase-3 enhanced by wortmannin, we prepared the cleavage site-directed antiserum against Caspase-3 (anti-p17) (Figure 5A and B). It is possible to detect the activation of protease by

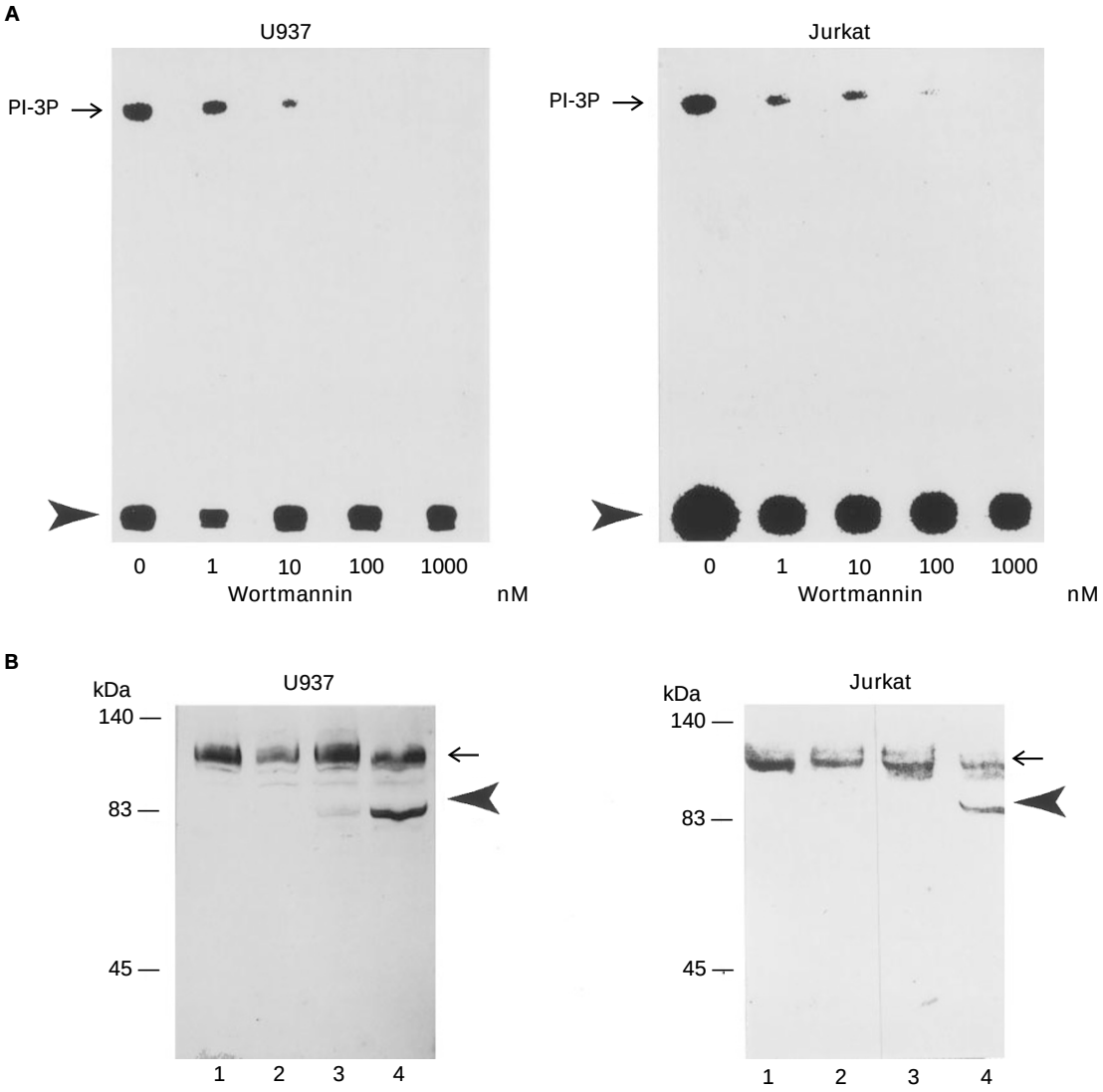

Figure 1 Effect of inhibition of PI-3K on the cleavage of PARP induced by TNF or anti-Fas. (**A**) Effect of wortmannin on the PI-3K activity of U937 cells or Jurkat cells. After U937 cells or Jurkat cells were incubated with wortmannin (1 – 1000 nM) for 20 min, PI-3K activity was assayed. Arrowheads indicate origin and arrows indicate phosphatidylinositol-3 phosphate. (**B**) Immunoblot analysis of the effect of wortmannin on the cleavage of PARP in TNF-treated U937 cells or anti-Fas-treated Jurkat cells. Left panel; U937 cells were incubated with or without TNF (50 U/ml) in the presence or absence of wortmannin (100 nM) for 5 h. Right panel; Jurkat cells were incubated with or without anti-Fas (25 ng/ml) in the presence or absence of wortmannin (100 nM) for 5 h. Lane 1, untreated cells; lane 2, wortmannin (100 nM); lane 3, TNF (50 U/ml) or anti-Fas (25 ng/ml); lane 4, TNF (50 U/ml) or anti-Fas (25 ng/ml) and wortmannin

the cleavage site-directed antiserum against target molecules (Saido *et al*, 1993). Activation of calpain, which is induced by its autoprocessing, can also be detected by the antiserum against cleavage site of calpain (Kikuchi and Imajoh-Ohmi, 1995). Caspase-3 is activated by processing of the inactive form (32K) into the active form (17K and

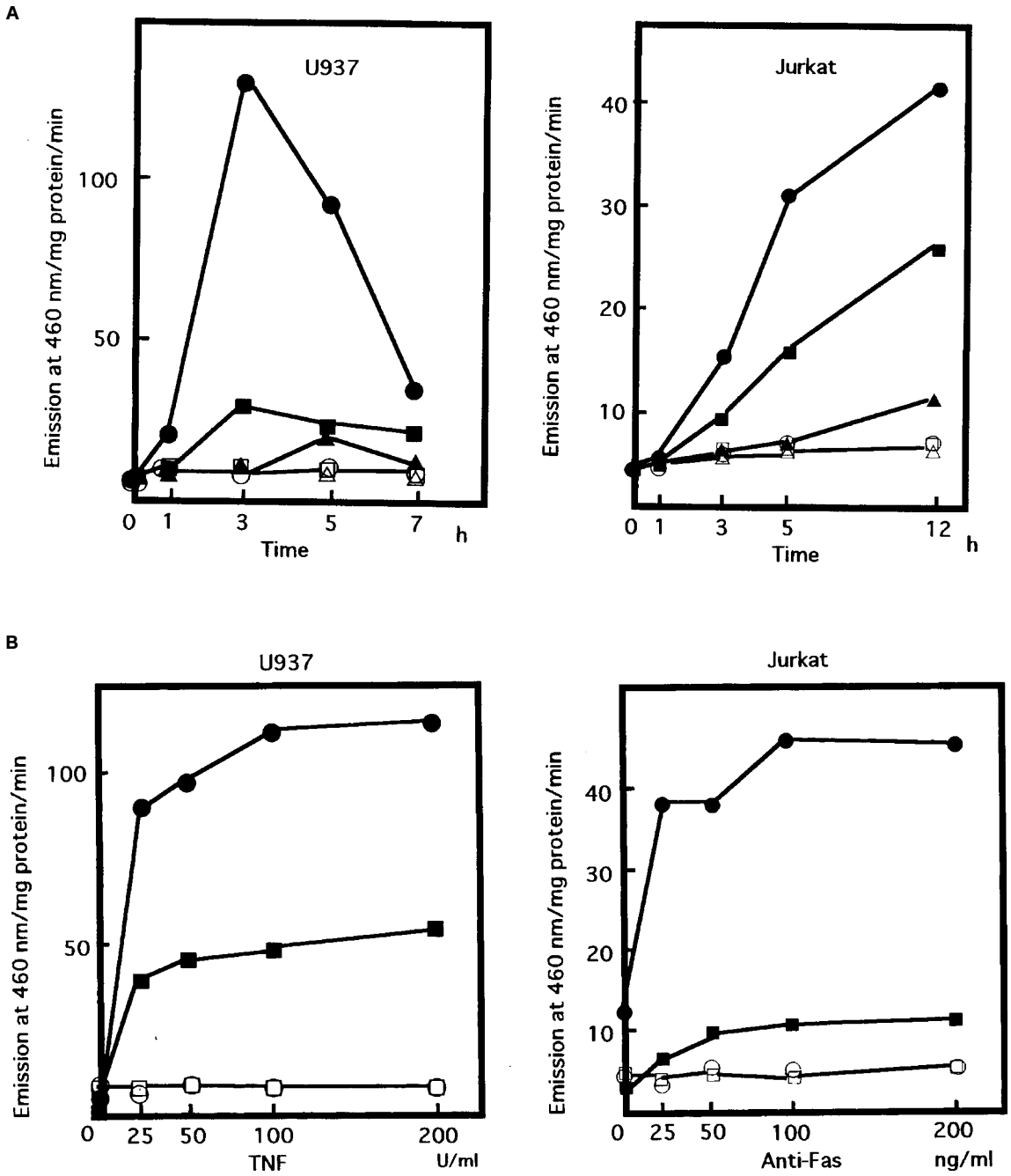

Figure 2 Effect of wortmannin on the Caspase-3-like protease activity of TNF-treated U937 cells or anti-Fas-treated Jurkat cells. (**A**) Time-dependent activation of Caspase-3-like and Caspase-1-like proteases by TNF (or anti-Fas) and wortmannin. Left panel; U937 cells were incubated with TNF (50 U/ml) in the presence or absence of wortmannin (100 nM) for indicated periods. Right panel; Jurkat cells were incubated with anti-Fas (25 ng/ml) in the presence or absence of wortmannin (100 nM) for indicated periods. Circles, squares and triangles represent cells treated with wortmannin and TNF (or anti-Fas), cells treated with TNF (or anti-Fas) alone and cells treated with wortmannin alone, respectively. Closed ones and open ones represent Caspase-3-like and Caspase-1-like activities, respectively. (**B**) Effect of wortmannin on the dose-dependent activation of Caspase-3-like protease by TNF or anti-Fas. Left panel; U937 cells were incubated with TNF at various concentrations in the presence or absence of wortmannin (100 nM) for 3 h. Right panel; Jurkat cells were incubated with anti-Fas at various concentration in the presence or absence of wortmannin (100 nM) for 7 h. Circles and squares represent cells treated with TNF (or anti-Fas) and wortmannin and cells treated with TNF (or anti-Fas) alone, respectively. Closed ones and open ones represent Caspase-3-like and Caspase-1-like activities, respectively

12K) during apoptosis induced by anti-Fas (Nicholson *et al*, 1995; Schlegel *et al*, 1996). The anti-p17 specifically reacts with the pentapeptide of cleavage site of Caspase-3 only after its cleavage (Figure 5A and B). Active fragment (p17) of Caspase-3 in TNF- or anti-Fas-treated cells was dose-dependently increased by wortmannin (1 – 100 nM) (Figure 6A and B), clearly showing that wortmannin enhanced the processing of Caspase-3 induced by TNF or anti-Fas.

The molecular mechanism, by which inhibition of PI-3K enhanced the cleavage of Caspase-3 in the TNF- or anti-Fas-treated cells, is not yet clear. One of the possible mechanisms is that inhibition of PI-3K or that of protein kinase at the downstream of PI-3K stimulates the activity of the processing enzyme of Caspase-3 in the TNF- or anti-Fas-treated cells. Caspase-1-like protease and Caspase-3-like protease are sequentially activated during anti-Fas-

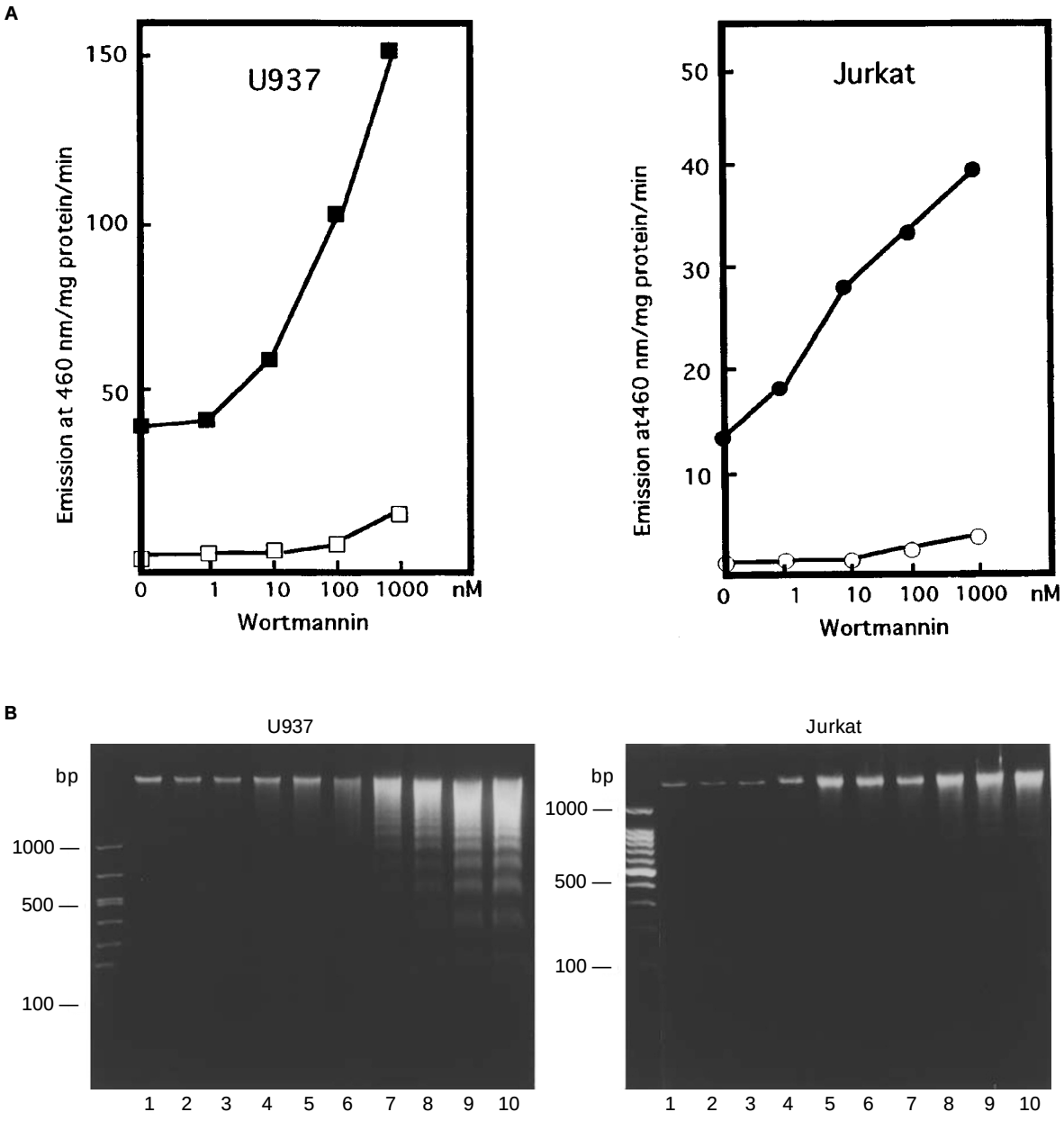

Figure 3 Dose-dependent effect of wortmannin on the Caspase-3-like activity and DNA fragmentation of TNF-treated U937 cells and anti-Fas-treated Jurkat cells. (**A**) Dose-dependent effect of wortmannin on the Caspase-3-like activity. Left panel; U937 cells were incubated with various concentration of wortmannin in the presence or absence of TNF (50 U/ml) for 3 h. Right panel; Jurkat cells were incubated with various concentration of wortmannin in the presence or absence of anti-Fas (25 ng/ml) for 7 h. Open squares and circles represent U937 cells and Jurkat cells in the presence of various concentration of wortmannin, respectively. Closed squares and circles represent the TNF-treated U937 cells and anti-Fas-treated Jurkat cells in the presence of various concentration of wortmannin, respectively. (**B**) Dose-dependent effect of wortmannin on the DNA fragmentation. Lanes 1 and 6; without wortmannin, lanes 2 and 7; with 1 nM wortmannin, lanes 3 and 8; with 10 nM wortmannin; lanes 4 and 9; with 100 nM wortmannin; lanes 5 and 10, with 1000 nM wortmannin; lanes 6 – 10 (left panel), with TNF(50 U/ml); lanes 6 – 10 (right panel), with anti-Fas (25 ng/ml)

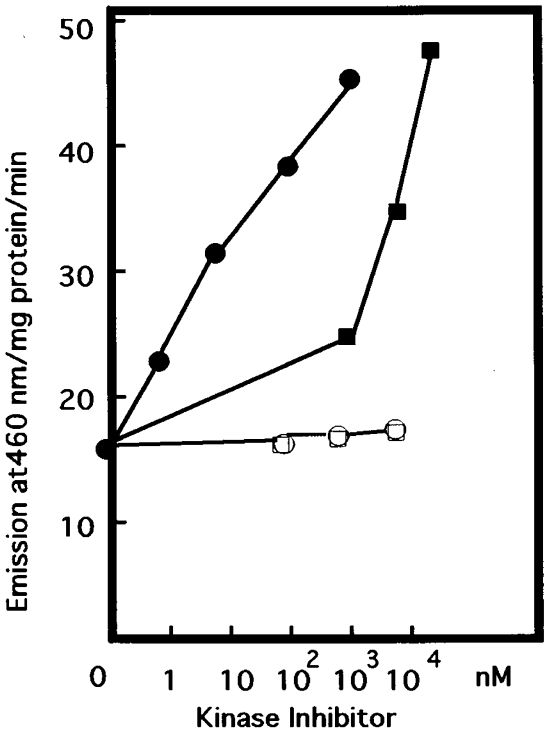

Figure 4 Effects of protein kinase inhibitors on the Caspase-3-like activity in the Fas-treated Jurkat cells. Effects of various kinase inhibitors on the Caspase-3-like activity in the Jurkat cells treated with anti-Fas (25 ng/ml) for 7 h. Closed circles, closed squares, open circles and open squares represent anti-Fas treated Jurkat cells in the presence of various concentration of wortmannin, LY294002, ML-9 (myosin light chain kinase inhibitor) and KN-93 (calmodulin dependent kinase inhibitor), respectively

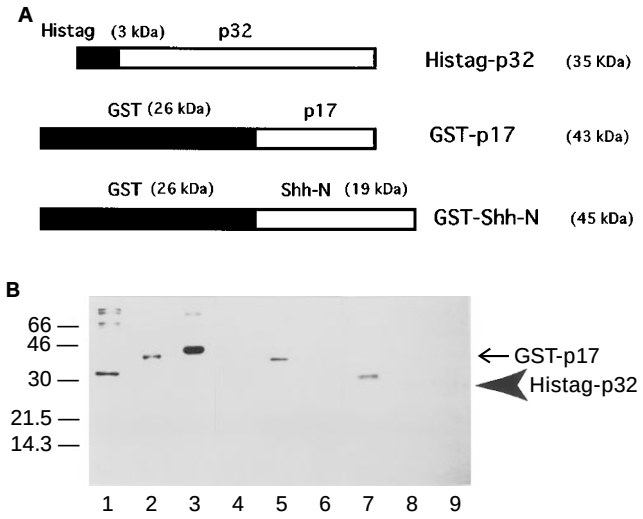

Figure 5 Reactivity of cleavage site-directed anti-serum for Caspase-3 (anti-p17). (**A**) Fusion protein of p17 and p32 of Caspase-3. To examine whether anti-p17 specifically reacts with cleaved fragment (p17), but not proCaspase-3 (p32), we prepared fusion protein of p17 and p32 of Caspase-3 as GST and Histag fusion proteins, respectively. GST-Shh-N (N-terminal portion of sonic hedgehog) was used as negative control. (**B**) Immunoblot analysis of anti-p17. The reactivity of anti-p17 was examined by immunoblot analysis. Anti-p32 was used for the detection of p32 of proCaspase-3. 1, 4, and 7, Histag-p32; 2, 5, and 8, GST-p17; 3, 6, and 9, GST-Shh-N. 1–3, Histag-p32 (1 μg), GST-p17 (1 μg) and GST-Shh-N (2 μg) were stained with Coommasie brilliant blue; 4–6, Histag-p32 (10 ng), GST-p17 (10 ng) and GST-Shh-N (20 ng) reacted with anti-p17; 7–9, Histag-p32 (10 ng), GST-p17 (10 ng) and GST-Shh-N (20 ng) reacted with anti-p32

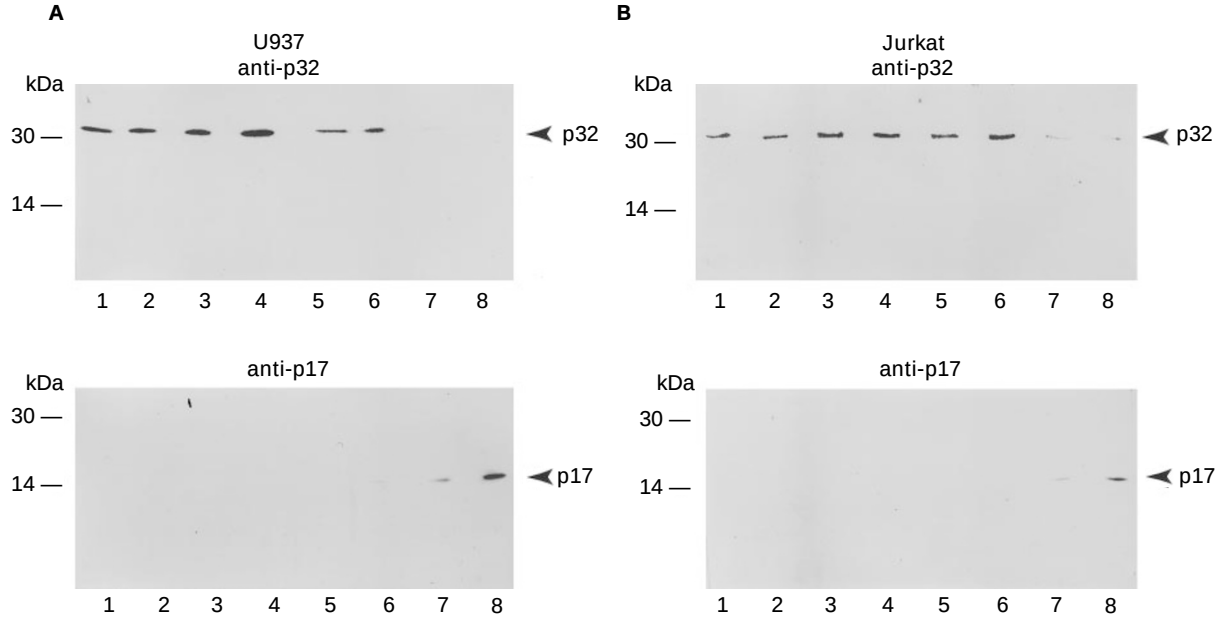

Figure 6 Dose-dependent effect of wortmannin on the cleavage of Caspase-3 of the TNF-treated U937 cells and anti-Fas-treated Jurkat cells. Effect of wortmannin on the cleavage of Caspase-3. (**A**) U937 cells were treated with wortmannin (1–100 nM) in the presence or absence of TNF (50 U/ml) for 3 h. (**B**) Jurkat cells were treated with wortmannin (1–100 nM) in the presence or absence of anti-Fas (25 ng/ml) for 7 h. Upper panel and lower panel are immunoblot analysis using anti-p32 and anti-p17, respectively. Lane 1, untreated cells; lanes 2 and 6, 1 nM wortmannin; lanes 3 and 7, 10 nM wortmannin; lanes 4 and 8, 100 nM wortmannin; Left panel, lanes 1–4 without TNF; lanes 5–8, with TNF; Right panel, lanes 1–4 without anti-Fas; lanes 5–8 with anti-Fas

induced apoptosis (Enari *et al*, 1996). Caspase-1 has an ability to cleave Caspase-3 to its active form *in vitro* (Tewari *et al*, 1995). However, PI-3K inhibitors enhanced the activation of Caspase-3 without any elevation of Caspase-1-like activity (Figure 2), suggesting that enhancement of the cleavage of Caspase-3 by wortmannin is independent on the activation of Caspase-1-like protease. On the other hand, Caspase-8, upstream of Caspase-3 in the Fas- or TNFR1-mediated death signal pathway, has an ability to process Caspase-3 into active form (Muzio *et al*, 1996). Since the cleavage of Ac-DEVD-MCA was slightly, but significantly, induced by wortmannin alone (Figure 3A), Caspases with Ac-DEVD-MCA cleavage activity such as Caspase-8 may be a processing enzyme activated by wortmannin.

The other possible mechanism is that wortmannin increases the susceptibility of Caspase-3 to the processing enzyme. Since wortmannin alone did not induce the cleavage of Caspase-3 at 1–100 nM, inhibition of PI-3K may not directly activate the processing enzyme. Recently it has been shown that Ced-4, upstream of Ced-3 and downstream of Ced-9, has a complex with not only Ced-9 and Ced-3 but also their mammalian homologues Bcl-2 and Caspase-3 or -8 (Chinnaiyan *et al*, 1997; Wu *et al*, 1997). Bcl-2 and Bcl-x_L suppress the activation of Caspase-3 and apoptosis induced by staurosporine (Chinnaiyan *et al*, 1996a; Armstrong *et al*, 1996). Survival factor IL-3 phosphorylates BAD and causes the loss of its death-promoting effects by inhibiting heterodimerization with Bcl-x_L (Zha *et al*, 1996; Wang *et al*, 1996). Thus PI-3K may block the susceptibility of Caspase-3 to the processing enzyme by regulating the phosphorylation of Bcl-2 family proteins and making a complex with Caspase-3, Bcl-2 and Ced-4.

Further study about the molecular mechanism of PI-3K regulating the activation of Caspase-3 remain to be studied.

Materials and Methods

Detection of cell death

U937 cells and Jurkat cells were cultured in RPMI-1640 medium containing 10% FCS in the CO_2 incubator at 37°C. Cells were incubated with wortmannin (100 nM) (Sigma, St. Louis, MO) in the presence or absence of TNF (Dainihonseiyaku, Osaka, Japan) or anti-Fas (MBL, Nagoya, Japan) for 5 h. Cell viability was determined by trypan blue exclusion. Values represent the average viability from three independent wells ($\pm$s.d.) and are normalized to the percentage of viable cells remaining in the untreated cultures. Two independent experiments showed similar results.

Isolation of fragmented DNA

U937 cells and Jurkat cells were respectively cultured with TNF and anti-Fas in the presence or absence of wortmannin at various concentrations (1–1000 nM). Cells were harvested, washed once with PBS, and used for DNA isolation. DNA isolation was accomplished according to the method described by Pringent *et al* (1993) with some modification. Four hundred μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 20 mM EDTA and 1% Triton X-100) was added to 2×10^6 cells and suspended. Lysis was allowed to proceed on ice for 10 min and the mixture was centrifuged for 10 min at 15 000 r.p.m. The supernatant was digested with 50 μg/ml RNase A at 37°C for 1 h. This was followed by a Proteinase K digestion at 50 μg/ml, at 37°C for 1 h. After phenol-chloroform extraction, the DNA was precipitated by adding 2.5 vol. of 100% ethanol and CH_3COONa (0.3 M at final concentration). DNA ladders could be visualized on 1.8% agarose gel.

Caspase-3-like and Caspase-1-like activity

U937 cells and Jurkat cells were respectively incubated with TNF and anti-Fas at various concentrations in the presence of various kinase inhibitors such as wortmannin, LY294002 (Funakoshi, Tokyo), ML-9 and KN-93 (Seikagaku Co., Tokyo) for indicated periods. After incubation, cells were washed two times with PBS, and the cell pellets were lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. After centrifugation at 10 000 $\times g$ for 5 min, the cell extracts (50 μg protein) were incubated with 10 μM Ac-DEVD-MCA (Peptide Institute, Osaka), synthetic peptide of PARP, or Ac-YVAD-MCA (Peptide Institute, Osaka), synthetic peptide of pro IL-1β, in the incubation buffer (50 mM Tris-HCl pH 7.5, 1 mM DDT) for 20 min in order to measure Caspase-3-like or Caspase-1-like activities, respectively. The reactions were halted by the addition of 10% SDS. The fluorescence was measured at 380 nm for excitation and at 460 nm for emission.

Assay of PI-3K

PI-3K activity was assayed according to the procedure as described previously (Arcaro and Wymann, 1993). U937 and Jurkat cells were incubated with wortmannin (Sigma, St. Louis, MO) at various concentration (1–1000 nM) and were lysed with lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 137 mM NaCl, 1 mM Na_3VO_4, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin). After centrifugation at 13 000 $\times g$ for 10 min, supernatant was incubated with anti-PI-3K (p85) (MBL, Nagoya) at 4°C overnight. Immuno-complex was precipitated with a mixture of protein G and A, and suspended with 20 μl of 20 mM HEPES buffer (pH 7.4). The immuno-complex was incubated in the reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 μM ATP, 200 μg/ml phosphatidylinositol, 200 μg/ml phosphatidylserine, 1 μCi γ-[^{32}P]ATP) at 25°C for 10 min and then subjected to thin layer chromatography. Thin layer plate (Merck, Darmstadt, Germany) was developed with chloroform/methanol/25% ammonia/H_2O (86: 76: 10: 14). After development, thin layer plate was exposed to Fuji image analyzer.

Preparation of anti-p17

The antiserum against p17, active fragment (17K) of Caspase-3, was prepared according to a manual for the preparation of a cleavage site-directed antibody against calpain (Saido *et al*, 1993; Kikuchi and Imajoh-Ohmi, 1995). Anti-p17 was raised by injecting a synthetic pentapeptide of the cleavage site of human Caspase-3, GIETD, conjugated with KHL into rabbits. Anti-p17 antibody was purified by peptide affinity column chromatography. The cDNA fragments encoding the p32 (inactive form of Caspase-3) and p17 were amplified by PCR using the following primers, respectively: p32, 5′-GACATATG-GAGAACACTGAAAACTC-3′ and 5′-GACATATGTTAGTGATAAAAA-TAG; p17, 5′-ATGGGAATATCCCTGGACAAC-3′ and 5′-CTAGTCTGTCTCAATGCCACA-3′. The PCR products were then cloned into the pCRII vector using the TA cloning kit (Invitrogen, San Diego, CA). Complement DNAs of p32 and p17 were subcloned in-frame into the *Nde*I and *Eco*RI site of the bacterial expression vectors of pET15 (Novagen, Madison, WI) and pGEX-4T-2 (Pharmacia Biotech,

Inc. Milwaukee, WI) for the preparation of Histag-p32 and GST-p17 fusion proteins, respectively. GST-p17 and Histag-p32 were purified by Glutathione and Nickel affinity column chromatography according to the manufacture's protocol, respectively. Purified fusion proteins were used for examining this specificity of anti-p17 by immunoblot analysis. GST-fusion protein of N-terminal region of rat sonic hedgehog (Shh-N) was used as a negative control (Urase *et al*, 1996).

Immunoblot analysis

Cleavage of PARP and Caspase-3 was examined by immunoblot analysis. U937 cells and Jurkat cells were respectively incubated with TNF and anti-Fas in the presence or absence of PI-3K inhibitors for indicated period. For the extraction of Caspase-3, the cell pellets were lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. For the extraction of PARP, the cell pellets were lysed with PBS containing 1% SDS and sonicated. After centrifugation at 10 000 $\times$ *g* for 5 min, the cell extracts (40 μg protein) were subjected to the SDS acrylamide (12% or 15%) gel electrophoresis and immunoblot analysis using anti-p32 (Transduction Laboratories, Lexington, KY), anti-p17 and anti-PARP. The reactivities with anti-PARP, anti-p32 and anti-p17 on the filters were detected by peroxidase-conjugated goat anti-mouse and anti-rabbit immunoglobulin (ABC kit, Vector Lab., Burlingame, CA), respectively.

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