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Differentiation-inducing factor-1-induced growth arrest of K562 leukemia cells involves the reduction of ERK1/2 activity

Emi Akaishi[a], Torao Narita[b], Shinjiro Kawai[c], Yoshikazu Miwa[d], Toshiyuki Sasaguri[d], Kohei Hosaka[a], Yuzuru Kubohara[b,*]

[a] *Department of Basic Sciences for Medicine, Gunma University School of Health Sciences, Maebashi 371-8514, Japan*
[b] *Institute for Molecular and Cellular Regulation (IMCR), Gunma University, 3-39-15 Showa-machi, Maebashi, Gunma-ken 371-8512, Japan*
[c] *Department of Biology, Osaka Dental University, Hirakata 573-1121, Japan*
[d] *Department of Clinical Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*

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Abstract

The differentiation-inducing factor-1 (DIF-1) is a signal molecule that induces stalk cell differentiation in the cellular slime mold *Dictyostelium discoideum*. In addition, DIF-1 is a potent antileukemic agent that induces growth arrest in K562 cells. In this study, we investigated the mechanism of action of DIF-1 in K562 cells in the light of cell-cycle regulators such as cyclins, retinoblastoma protein (pRb), and the mitogen-activated protein kinase (MAPK) family. DIF-1 down-regulated cyclins D/E and a phosphorylated form of pRb (p-pRb), and thereby induced G_1 arrest of the cell cycle. DIF-1 inactivated the extracellular signal-regulated kinase (ERK) in a biphasic manner but did not affect the c-Jun N-terminal kinase (JNK) or p38 MAPK. The MEK (MAPK kinase) inhibitor, U0126, which has been shown to induce growth arrest, inactivated ERK and down-regulated cyclins D and E. Although DIF-1 activated the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway, neither wortmannin nor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002; PI-3K inhibitors) cancelled DIF-1-induced growth arrest. The present results suggest that ERK inactivation may be involved in DIF-1-induced growth arrest and that PI-3K activity is not required for DIF-1-induced growth arrest in K562 cells.

Keywords: *Dictyostelium*; DIF-1 (differentiation-inducing factor-1); K562; ERK (extracellular signal-regulated kinase); Akt

1. Introduction

The differentiation-inducing factor-1 (DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one) (Fig. 1) is a lipophilic signal molecule that induces stalk cell differentiation in the cellular slime mold *Dictyostelium discoideum* (Morris et al., 1987; Kay et al., 1989). DIF-1 is thought to function, at least in part, via an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (Kubohara and Okamoto, 1994; Schaap et al., 1996; Azhar et al., 1997), but the precise signaling system of DIF-1, including the target molecule(s) of DIF-1, is still unknown.

On the other hand, it has been shown that DIF-1 exhibits antitumor activities, inhibition of cell growth (G_1 arrest), induction or promotion of cell differentiation, and induction of apoptosis, depending on the concentrations of DIF-1 (Asahi et al., 1995; Kubohara et al., 1995a,b; Kubohara, 1997, 1999). Of the DIF analogs tested so far, DIF-3 (Fig. 1), a metabolite of DIF-1 in *D. discoideum*, is the most potent antitumor agent (Kubohara, 1999). As to the mechanism of the action of DIF (DIF-1 and DIF-3), we have found that DIF increases $[Ca^{2+}]_i$ in some tumor cells (Kubohara et al., 1995a,b; Kubohara, 1997, 1999) and activates Akt/protein kinase B (PKB) in human leukemia K562 cells (Kubohara and Hosaka, 1999). In vascular smooth muscle cells, DIF-1 inhibits the expression of cyclins D and E and the phosphorylation of retinoblastoma protein (pRb), thereby arresting the cell cycle at G_1 and inducing smooth muscle cell differentiation in vitro (Miwa

* Corresponding author. Tel.: +81-27-220-8866; fax: +81-27-220-8897.
E-mail address: kubohara@showa.gunma-u.ac.jp (Y. Kubohara).

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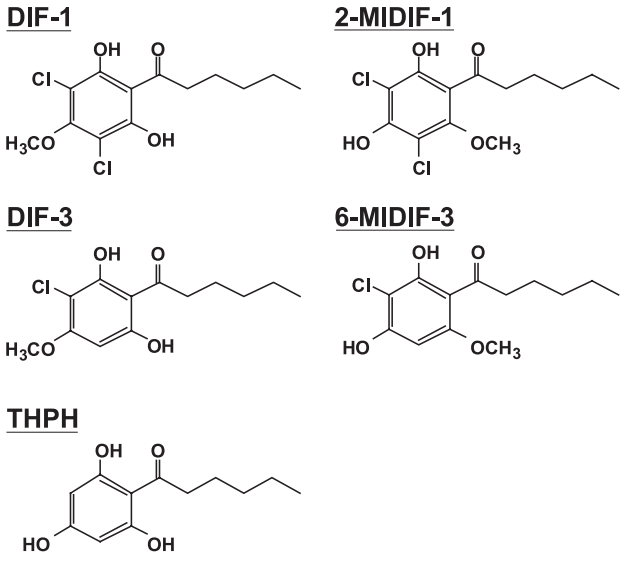

Fig. 1. Chemical structure of DIF analogs. DIF-1 = 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one; DIF-3 = 1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one; 2-MIDIF-1 = 2-methoxy isomer of DIF-1; 6-MIDIF-3 = 6-methoxy isomer of DIF-3; THPH = 1-(2,4,6-trihydroxyphenyl)hexan-1-one.

et al., 2000). Yet, the mechanisms underlying the actions of DIF remain to be elucidated.

The mitogen-activated protein kinase (MAPK) family, which includes extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK (p38), regulates a variety of cellular events such as cell proliferation and differentiation (Cano and Mahadevan, 1995; Seger and Krebs, 1995; Lewis et al., 1998; Nagata et al., 1998). It has been reported that the MAPK family is involved in erythroid differentiation induced by butyrate, hydroxyurea, or hemin in K562 cells (Rivero and Adunyah, 1996; Witt et al., 2000; Park et al., 2001; Woessmann and Mivechi, 2001). Furthermore, U0126 and PD98059, inhibitors of MEK (MAPK kinase), have been shown to induce growth arrest and erythroid differentiation in K562 cells (Kang et al., 1999; Witt et al., 2000; Park et al., 2001; Woessmann and Mivechi, 2001). In contrast, sustained activation of ERK may be required for megakaryocytic differentiation in K562 cells (Racke et al., 1997; Whalen et al., 1997; Herrera et al., 1998).

In the present study, we investigated the mechanism by which DIF-1 induces growth arrest in K562 leukemia cells with respect to the role of the MAPK family. We show here that DIF-1 may induce growth arrest via inactivation of ERK and downregulation of G_1 cyclins. Furthermore, since it has been shown that DIF-1 activates the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway in this cell line (Kubohara, 1999; Kubohara and Hosaka, 1999), the involvement of PI-3K/Akt was also assessed. It is shown that PI-3K/Akt may not be involved in DIF-1-induced growth arrest.

2. Materials and methods

2.1. Cell line and reagents

Human leukemia K562 cells were used in this study. DIF-1 was purchased from Affiniti Research Products (Exeter, UK). A 2-methoxy isomer of DIF-1 (2-MIDIF-1), DIF-3, a 6-methoxy isomer of DIF-3 (6-MIDIF-3), and 1-(2,4,6-trihydroxyphenyl)hexan-1-one (THPH) were synthesized by a chemical company following the method of Masento et al. (1988). All the DIF analogs were stored at −20 °C as 10 mM solutions in ethanol. Wortmannin and Alamar Blue (cell number indicator) were purchased from Wako (Osaka, Japan). 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) was from Sigma (St. Louis, MO). Wortmannin and LY294002 were stored at −20 °C as 0.1 mM solution in ethanol and 10 mM solution in dimethylsulfoxide (DMSO), respectively. Rabbit anti-Akt, anti-phospho-Akt (Ser^{473}), anti-p38 MAPK, and anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibodies were bought from New England BioLabs (Beverly, MA). Rabbit anti-SAPK/JNK antibody and anti-phosho-GSK-3β (Ser^{9}) were from Cell Signaling Technology (Beverly, MA). Anti-Rb protein monoclonal antibody was from MBL (Nagoya, Japan), anti-cyclin D_3 antibody was from Transduction Laboratories (Lexington, KY), and rabbit anti-cyclin D_1 (M-20), anti-cyclin E (C-19), anti-Cdk2 (M2), and anti-Cdk4 (H-22) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Two different anticyclin D_2 monoclonal antibodies were from Sigma and BD PharMingen (San Diego, CA). Rabbit anti-Erk1/2, anti-active MAPK, and antiactive JNK antibodies were obtained from Promega (Madison, WI). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies were from Amersham (UK).

2.2. Cell culture and immunoblotting (Western analysis)

K562 cells were maintained at 37 °C (5% CO_2) in tissue culture dishes filled with a growth medium (an RPMI 1640 medium with 10% fetal bovine serum, 25 μg/ml penicillin, and 50 μg/ml streptomycin—designated RPMI).

For immunoblotting, cells were incubated in 90-mm culture dishes filled with 10 ml of RPMI (at $1–3 \times 10^5$ cells/ml) containing various reagents. At appropriate time points, they were collected by centrifugation ($400 \times g$, 3–4 min), washed with a Tris buffer (a 10 mM Tris–HCl buffer, pH 7.2, 137 mM NaCl, and 2.7 mM KCl), and lysed with a sodium dodecyl sulfate (SDS) sample buffer ($1–2 \times 10^4$ cells/μl).

To observe the short-time effect of DIF-1, cells were suspended in RPMI supplemented with 20 mM HEPES/NaOH, pH 7.4, g in Eppendorf tubes (2×10^6 cells/ml/tube) and stimulated with 30 μM DIF-1 for 3–15 min on a twin mixer. The cells were collected by centrifugation (5000 rpm, 30 s) and lysed with an SDS sample buffer

(10^4 cells/μl). The cell proteins (10^5 cells/lane) were separated by SDS polyacrylamide gel electrophoresis (PAGE) (usually 10% polyacrylamide gel was used, and 7.5% gel was used for the blot for Rb protein) and transferred onto nitrocellulose membranes. The membrane was incubated with a primary antibody and then with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody as described previously (Kubohara et al., 2003). The membrane was processed for visualization using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham) and exposed to Hyperfilm for ECL (Amersham). The visualized bands in the ECL image were digitized and quantified using Adobe Photoshop and NIH Image software.

2.3. Northern analysis

Cells were incubated in 9-cm dishes, each containing 10 ml of RPMI (5×10^5 cells/ml) in the presence of 20 μM DIF-1 or DIF-3. Cells were harvested by centrifugation, and RNAs were prepared from them by the use of TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. RNAs were used for Northern hybridization as described previously (Sasaguri et al., 1996; Ishida et al., 1997). The visualized bands were digitized and quantified using the Adobe Photoshop and NIH Image softwares.

2.4. FACS analysis

Cells were incubated for 24 h in 90-mm culture dishes filled with 10 ml f RPMI (at $2–3 \times 10^5$ cells/ml) in the presence or absence of 30 μM DIF-1. They were harvested by centrifugation ($400 \times g$, 3 min), suspended in 10 ml of cold 75% ethanol, and stored at 4 °C overnight. They were analyzed for DNA contents by a flow cytometer as described previously (Kubohara, 1997).

2.5. Assay for cell growth

Cells were incubated in a multi(12)-well plate, each well containing 1 ml of RPMI (5×10^4 cells/ml) in the presence or absence of DIF analogs and/or wortmannin or LY294002. On day 3, a 1/20 vol (50 μl) of Alamar Blue (cell number indicator) was added to each well, and after 1–2 h of incubation at 37 °C (5–8% CO_2), 150 μl of each of the sample solutions was transferred into a 96-well plate, and absorbance at 570 nm (reference at 595 nm) was measured with a microplate reader (Model 550; Bio-Rad). A cell number was given as a ratio of the absorbance (percent of control).

2.6. Measurement of DNA synthesis

DNA synthesis was observed by measuring 5-bromo-2′ deoxy-uridine (BrdU) incorporation using the BrdU labeling and detection kit III (Boehringer Mannheim, Indianapolis, IN). Briefly, K562 cells were incubated for 2 h at 37 °C (5% CO_2) in a multi(96)-well plate (4×10^4 cells/100 μl/well) in RPMI with or without DIF-1 and/or wortmannin. Prior to cell incubation, the wells were coated with poly-D-lysine; 50 μl of poly-D-lysine (0.2 mg/ml) solution was added to each well and incubated for 1 h at room temperature. The poly-D-lysine solution was aspirat-

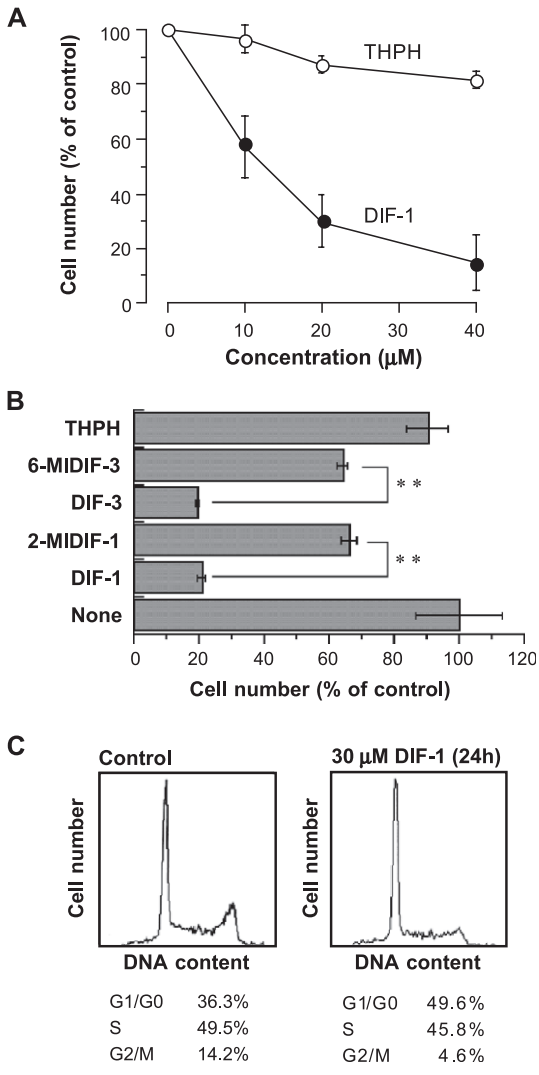

Fig. 2. Effects of DIF on cell growth in K562 cells. (A) K562 cells were incubated with or without 10–40 μM DIF-1 or THPH. On day 3, the relative cell number was optically measured with the cell number indicator, Alamar Blue, as described in Materials and Methods. Data are given with the mean ± S.D. of three independent experiments ($n = 3$) (the mean of control cell number = 100). (B) Cells were incubated with or without 30 μM DIF analogs. On day 3, the relative cell number was optically measured with the cell number indicator, Alamar Blue. Triplicate determination was performed for each analog, and the mean ± S.D. values ($n = 3$) are shown. $^{**}P < 0.0001$ (by ANOVA: post-hoc Fisher's PLSD). (C) Cells were incubated for 24 h in the presence or absence of 30 μM DIF-1 and fixed with ethanol. They were analyzed for DNA contents by a flow cytometer, and the cell cycle phase distribution was estimated as described previously (Kubohara, 1997).

ed, and the wells were washed with RPMI and used for cell incubation. Ten microliters of BrdU labeling reagent was added to each well and incubated for 2 h at 37 °C (5% CO_2). Cells were precipitated by centrifugation (350 × *g*, 10 min), and the supernatants were carefully aspirated. Cells were fixed with 70% ethanol (0.5 M HCl) and used for the assay for BrdU incorporation according to the manual. Finally, the quantity of BrdU taken up by the cells was detected by measuring absorbance at 405 nm (reference at 492 nm) with a microplate reader.

3. Results

3.1. Effects of DIF on cell growth in K562 cells

We first examined the effects of DIF-1 and its analogs (Fig. 1) on cell growth in K562 cells. As shown in our previous papers (Kubohara, 1999; Kubohara and Hosaka, 1999), DIF-1 suppressed cell growth in a dose-dependent manner, but THPH at up to 40 μM showed no marked effect (Fig. 2A). 2-MIDIF-1 or 6-MIDIF-3 at 30 μM did not suppress cell growth as strongly as DIF-1 or DIF-3 (Fig. 2B), indicating that the effects of DIF (DIF-1 and DIF-3) are chemical structure-specific and that there may be the target molecule(s) for DIF in the cells. FACS analysis revealed that DIF-1 induced G_1 arrest (Fig. 2C).

To investigate how DIF-1 induces G_1 arrest, we examined the effects of DIF-1 on cell cycle-regulating proteins in K562 cells (Fig. 3). Northern analysis revealed that DIF-1 reduced the expression of cyclin D_1 and E mRNA (Fig. 3A), which was confirmed by DNA chip analysis (data not shown). Cyclin G_2 was upregulated under the same conditions (Fig. 3A), which agrees well with the previous notion that cyclin G_2 may be a negative regulator of cell cycle progression (Horne et al., 1997). Quite similar results were obtained with DIF-3 (Fig. 3A), which is the most potent antileukemic agent tested so far (Kubohara, 1999).

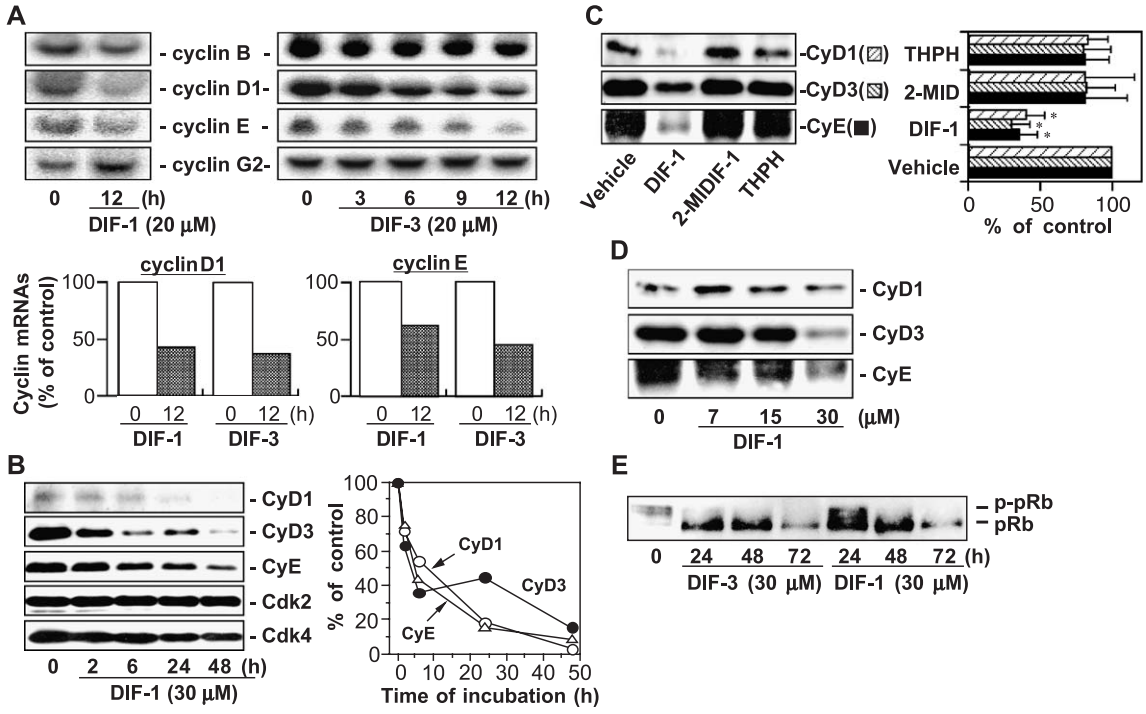

Fig. 3. Effects of DIF on the expression of cyclins, Cdks, and Rb proteins in K562 cells. (A) Cells were incubated with 20 μM DIF-1 or DIF-3 and harvested at the indicated time points. RNAs were prepared from them, and Northern analysis (10 μg RNA/lane) was performed with the indicated probes. Cyclin B, D_1, and E mRNAs were down-regulated, and cyclin G mRNA was up-regulated in the presence of DIF-1. The bands for cyclin D_1 and E mRNAs were quantified and presented as percent of control (graphs). (B) Cells were incubated for the indicated times with 30 μM DIF-1, and cell proteins were analyzed by Western blot for cyclins D_1, D_3, and E and Cdk2/4. The bands for cyclins D_1, D_3, and E were quantified and presented as percent of the control (graph). Cyclins D and E were downregulated, but Cdk2 and Cdk4 were not significantly affected by DIF-1. Note that cyclin D_2 was not easily detected with two different antibodies for cyclin D_2 (data not shown). (C) Cells were incubated for 24 h with 30 μM DIF-1, 2-MIDIF-1, THPH, or vehicle (0.3% ethanol), and cell proteins were analyzed by Western blot for cyclins D_1, D_3, and E. The bands for cyclins were quantified as percent of control, and the mean ± S.D. values of three independent experiments are shown (graph). Cyclins D_1, D_3, and E were significantly reduced in the presence of DIF-1 (*$P < 0.02$ vs. others, by ANOVA). (D) Cells were incubated for 6 h with the indicated concentrations of DIF-1, and cell proteins were analyzed by Western blot for cyclins D_1, D_3, and E. (E) Cells were incubated for the indicated times with 30 μM DIF-1 or DIF-3, and cell proteins were analyzed by Western blot for Rb protein (*pRb*). Representative blots of three similar experiments are shown. A phosphorylated form of pRb (*p-pRb*) was induced to be a dephosphorylated form (*pRb*) along with the incubation with DIF-1 or DIF-3. It should be noted that dephosphorylation of p-pRb occurred with DIF-3 slightly earlier than with DIF-1.

Since cyclins are often regulated at the protein level by their production and proteolysis, it is important to observe their protein levels. Western analysis showed that DIF-1 greatly reduced the amounts of D-type cyclins and cyclin E (Fig. 3B) in a dose-dependent manner (Fig. 3D). DIF-1 had little effect on the levels of cyclin-dependent kinase 2 (Cdk2) and Cdk4 (Fig. 3B); these two are the kinases for the phosphorylation of the pRb. Importantly, two analogs of DIF-1, 2-MIDIF-1 and THPH, did not reduce cyclins D_1, D_3, and E (Fig. 3C), indicating that the effects of DIF-1 on the G_1 cyclins are chemical structure-specific. The phosphorylated form of pRb (designated p-pRb) was reduced along with incubation with DIF-1 or DIF-3, and most p-pRb were finally dephosphorylated (Fig. 3E). These results indicate that DIF may induce G_1 arrest, at least in part, by reducing the levels of cyclins D_1, D_3, and E, and thereby dephosphorylating p-pRb in K562 leukemia cells. These results are nearly consistent with our previous results in vascular smooth muscle cells (Miwa et al., 2000) and HeLa cells (Takahashi-Yanaga et al., 2003).

3.2. Effects of DIF-1 on MAP kinase family

The MAPK family regulates a variety of cellular events such as cell proliferation and differentiation (Cano and Mahadevan, 1995; Seger and Krebs, 1995; Lewis et al., 1998) and has been shown to be involved in erythroid differentiation induced by some reagents in K562 cells (Nagata et al., 1998; Rivero and Adunyah, 1996; Witt et al., 2000; Woessmann and Mivechi, 2001). Therefore, we examined the effects of DIF-1 on the MAPK family in K562 cells (Fig. 4).

DIF-1 at 30 μM did not have much effect on the protein levels of ERK, JNK, and p38 MAPK (p38) throughout the incubation period (Fig. 4A). Interestingly,

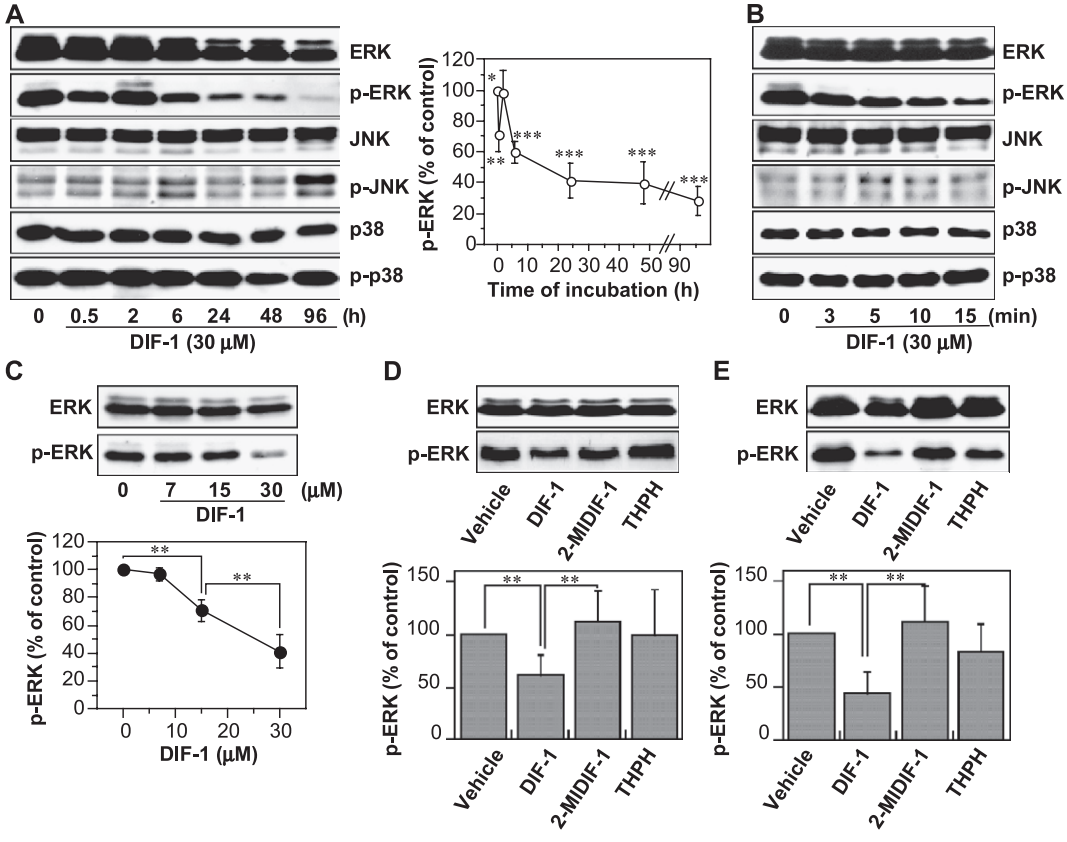

Fig. 4. Effects of DIF on the MAPK family in K562 cells. (A) Cells were incubated for the indicated times with 30 μM DIF-1 (at 37 °C), and cell proteins were analyzed by Western blot for ERK, phospho-ERK (*p-ERK*), JNK, phospho-JNK (*p-JNK*), p38 MAPK (*p38*), and phospho-p38 MAPK (*p-p38*). The bands of p-ERK in the blots were quantified, and data are given with the mean ± S.D. values of two independent experiments ($n=2$) (graph). $^{**}P<0.05$, $^{***}P<0.01$, compared with $^{*}t_0$ control (by ANOVA). (B) Cells in Eppendorf tubes were incubated for the indicated times with 30 μM DIF-1 (at room temperature), and cell proteins were analyzed by Western blot for the MAPK family. Similar results were obtained in another experiment. (C) Cells were incubated for 6 h (at 37 °C) with the indicated concentrations of DIF-1, and cell proteins were analyzed by Western blot for ERK and phospho-ERK (*p-ERK*). The bands of p-ERK in the blots were quantified, and data are given with the mean ± S.D. values of two independent experiments ($n=2$) (graph). $^{**}P<0.02$ (by ANOVA). (D, E) Cells were incubated (at 37 °C) for 6–10 min (D) or 24 h (E) with 0.3% ethanol (*vehicle*) or 30 μM DIF-1, 2-MIDIF-1, or THPH, and cell proteins were analyzed by Western blot. The bands for p-ERK in the blots were quantified, and data are given with the mean ± S.D. values of four (D) or three (E) independent experiments (graphs). $^{**}P<0.05$ (by ANOVA).

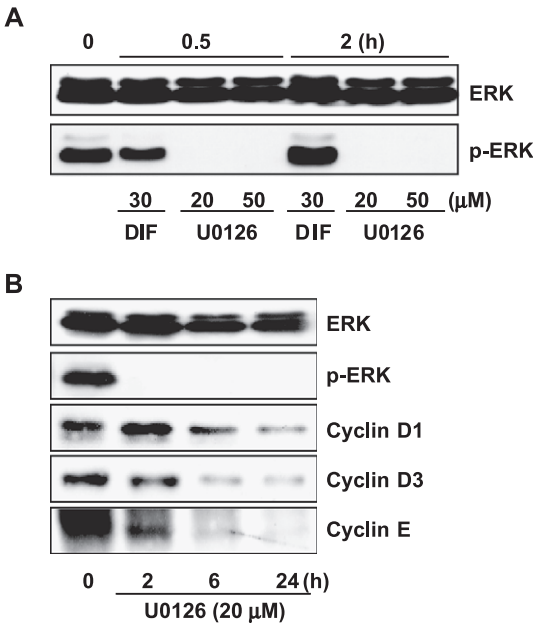

Fig. 5. Effects of U0126 on G_1 cyclins. (A) Cells were incubated with DIF-1 (30 μM) or U0126 (20 and 50 μM) for 0.5 and 2 h, and cell proteins were analyzed by Western blot for ERK and phospho-ERK (p-ERK). U0126 inhibited ERK well. (B) Cells were incubated with U0126 (20 μM) for the indicated times, and cell proteins were analyzed by Western blot for ERK, p-ERK, and cyclins D_1, D_3, and E.

however, DIF-1 reduced the ERK activity in a biphasic manner, that is, the phosphorylated (active) form of ERK decreased at 0.5 h of incubation with DIF-1, then increased at 2 h, and again decreased thereafter (Fig. 4A). DIF-1-induced reduction of ERK activity was observed within 15 min (Fig. 4B) and DIF-1 at 7–30 μM reduced ERK activity at 6 h in a dose-dependent manner (Fig. 4C). The activities of JNK and p38 did not change much during incubation with DIF-1 (Fig. 4A and B). Importantly, 2-MIDIF-1 or THPH did not have a significant effect on the activity of ERK at 6–10 min (Fig. 4D) and at 24 h (Fig. 4E). DIF-3 as well as DIF-1 inactivated ERK in a biphasic manner, while 6-MIDIF-3 only slightly reduced the activity of ERK (data not shown). These results agree well with the observation that DIF-1 and DIF-3 strongly suppressed cell growth, but their isomers and THPH did not (Fig. 2) (Kubohara, 1999).

3.3. Effects of MEK inhibitor (U0126) on G_1 cyclins

U0126 (a MEK inhibitor that inactivates ERK) induces growth arrest and erythroid differentiation in K562 cells (Witt et al., 2000; Woessmann and Mivechi, 2001); thus, we investigated if U0126 reduces G_1 cyclins (Fig. 5). ERK was indeed inactivated by U0126 at 20–50 μM, and inactivation of ERK by U0126 resulted in a reduction of cyclins D_1, D_3, and E (Fig. 5B). The results indicate that the mere inhibition of ERK may be sufficient to explain the action of DIF-1 in K562 cells.

3.4. Effects of PI-3K inhibitors on DIF-induced ERK inactivation and growth arrest

DIF-1 has been shown to induce the activation of PI-3K and Akt in K562 cells (Kubohara and Hosaka, 1999). To verify the involvement of PI-3K/Akt, we examined the effect of wortmannin, an inhibitor of PI-3K, on DIF-1-induced growth arrest (Fig. 6). However, wortmannin at 0.1 μM, which perfectly inhibited DIF-induced Akt phosphorylation (Fig. 7A), slightly inhibited DNA synthesis (Fig. 6A) and had no influence on the DIF-1-induced inhibition of DNA synthesis (Fig. 6A) and cell growth (Fig. 6B). LY294002 (long-term inhibitor of PI-3K) at 30 μM was inhibitory to cell growth by itself and did not affect the DIF-1-induced growth arrest (Fig. 6B). These results suggest that a basal activity of PI-3K is required for normal cell growth but that DIF-1-induced growth arrest may not involve the PI-3K/Akt pathway.

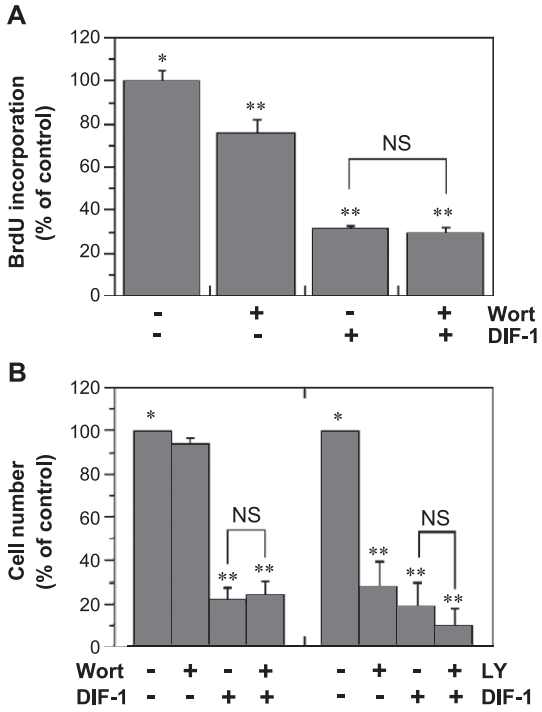

Fig. 6. Effects of PI-3 kinase inhibitors on DNA synthesis and cell growth in K562 cells. (A) Cells were incubated in 96-well plates with or without 30 μM DIF-1 and/or 0.1 μM wortmannin (*Wort*) for 2 h. BrdU was then added to each well and further incubated for 2 h. The cells were assayed for BrdU incorporation as described in Materials and Methods. Data are given with the mean ± S.D. ($n = 5$; five-well determination was performed) of relative absorbance at 405 nm (reference at 492 nm). Wortmannin at 0.1 μM was rather inhibitory to DNA synthesis and did not affect the inhibition of DNA synthesis by DIF-1. $^{**}P < 0.001$, compared with *control (by ANOVA). NS = not significant. (B) Cells were incubated with or without 30 μM DIF-1 and/or 0.3 μM wortmannin (*Wort*) or 30 μM LY294002 (*LY*) for 3 days, and the relative cell number was optically measured with Alamar Blue. Data (percent of control) are given with the mean ± S.D. of three independent experiments ($n = 3$). Wortmannin or LY294002 was inhibitory to cell growth and did not affect the inhibition of cell growth by DIF-1. $^{**}P < 0.001$, compared with *control (by ANOVA). NS = not significant.

To assess whether the PI-3K/Akt pathway is involved in the DIF-induced biphasic inactivation of ERK, we examined the effects of wortmannin and LY294002 on ERK activity in the presence of DIF-1 (Fig. 7). As expected (Kubohara and Hosaka, 1999), DIF-1 at 30 μM activated Akt, and wortmannin at 0.1 μM perfectly inhibited the DIF-induced Akt activation (Fig. 7A). Wortmannin alone did not affect the activity of the MAPK family, and the early-phase inactivation of ERK by DIF-1 was not cancelled by wortmannin (Fig. 7A). As indicated in Figs. 3 and 4, DIF-1 reduced the levels of p-ERK, and cyclins D_1, D_3, and E at 24 h (Fig. 7B). However, LY294002 did not cancel the DIF-1-induced reduction of the proteins and rather showed additive effects on the reduction of the proteins by DIF-1 (Fig. 7B). It should be noted that LY294002 by itself also reduced them (Fig. 7B), indicating that PI-3K is required for the ordinary activity of ERK and the maintenance of cyclins D_1, D_3, and E in the proliferating K562 cells. The present results suggest that PI-3K/Akt may not be involved in the process of DIF-induced reduction of p-ERK, and cyclins D_1, D_3, and E (Fig. 8) in spite of the fact that DIF-1 activates PI-3K/Akt (Fig. 7).

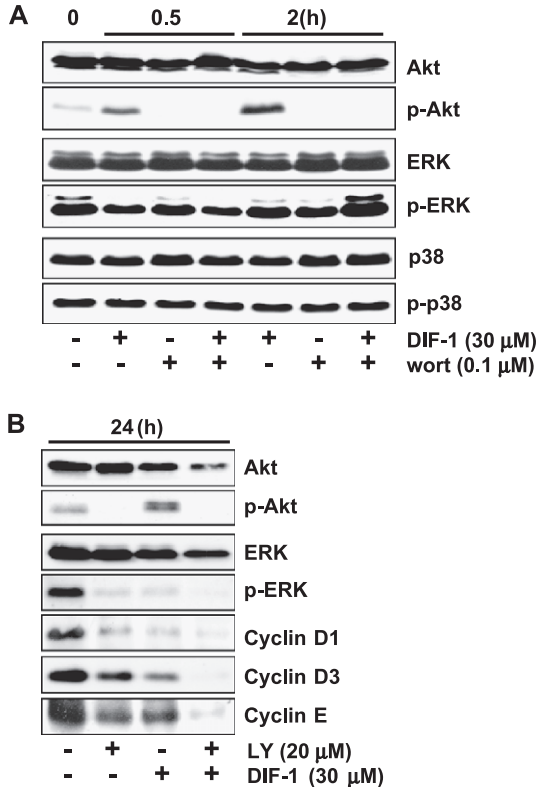

Fig. 7. Effects of PI-3 kinase inhibitors on the MAPK family in K562 cells. (A) Cells were incubated for the indicated times with or without 30 μM DIF-1 and/or 0.1 μM wortmannin (*wort*), and cell proteins were analyzed by Western blot for Akt, p-Akt, and the MAPK family. Representative blots of two similar experiments are shown. Wortmannin at 0.1 μM inhibited DIF-induced Akt activation but neither affected the MAPK family nor canceled DIF-induced ERK inactivation. (B) Cells were incubated without or with 30 μM DIF-1 ± 20 μM LY294002 (*LY*) for 24 h and analyzed by Western blot for Akt, p-Akt, ERK, p-ERK, and cyclins D_1, D_3, and E. All the samples contained both ethanol (0.3%) and DMSO (0.2%) as vehicles. Representative blots of three independent experiments are shown. Note that p-Akt is well abolished with LY294002 and that total Akt is downregulated in the presence of both DIF-1 and LY294002.

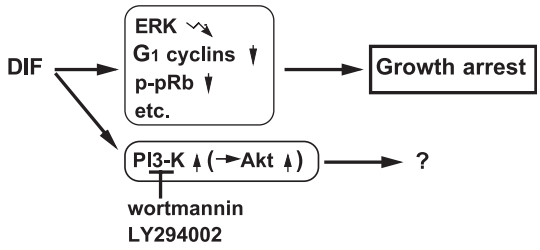

Fig. 8. Hypothetical scheme for the action of DIF in K562 cells. DIF downregulates G_1 cyclins and p-pRb (Fig. 3) and induces growth arrest (Fig. 2). Furthermore, DIF inactivates ERK in a biphasic manner but does not affect JNK or p38 MAPK (Fig. 4). Because the inactivation of ERK by U0126 or PD98059 can induce growth arrest (Witt et al., 2000; Park et al., 2001; Woessmann and Mivechi, 2001), it is probable that inactivation of ERK is involved in DIF-induced growth arrest. However, the significance of the biphasic inactivation of ERK by DIF is not known. On the other hand, DIF activates PI-3K and Akt (Fig. 7) (Kubohara and Hosaka, 1999). However, since the PI-3K inhibitors (wortmannin and LY294002) did not cancel the DIF-induced growth arrest (Fig. 6) and also because LY294002 itself was inhibitory to cell growth (Fig. 6B) and the expression of cyclins D_3 and E (Fig. 7B), activation of the PI-3K/Akt pathway may not be involved in DIF-induced growth arrest. The downstream cascades of PI-3K/Akt that are activated by DIF remain to be elucidated.

4. Discussion

We have previously shown that DIF (DIF-1 and DIF-3) suppresses cell growth and induces/promotes cell differentiation in mammalian cells such as mouse erythroleukemia B8, human leukemia K562 and HL-60, and vascular smooth muscle cells (Asahi et al., 1995; Kubohara et al., 1995a,b; Kubohara, 1997, 1999; Miwa et al., 2000). In the present study, we investigated the mechanism of action of DIF in K562 cells and found that DIF-1 reduced the activity of ERK. Furthermore, we investigated the relationship among PI-3K/Akt, ERK, and cell growth. The hypothetical scheme for the actions of DIF is presented in Fig. 8.

4.1. Involvement of ERK inactivation in DIF-induced growth arrest

The ERK cascade is a key regulator of cell proliferation and differentiation in a variety of cells (Cano and Mahadevan, 1995; Seger and Krebs, 1995; Lewis et al., 1998; Nagata et al., 1998). In K562 cells, it has been shown that sustained activation of ERK could be required for megakaryocytic differentiation and growth arrest, whereas transient activation of ERK leads to cell proliferation (Racke et al., 1997; Whalen et al., 1997; Herrera et al., 1998). In contrast, inactivation of ERK by U0126 or PD98059 results in growth arrest and erythroid differentiation (Kang et al.,

1999; Witt et al., 2000; Park et al., 2001; Woessmann and Mivechi, 2001).

In the present study, we found for the first time that DIF reduces ERK activity in a biphasic manner (Fig. 4). Although the significance and mechanism of the biphasic inactivation of ERK are not known, the biphasic inactivation of ERK could be the simple sum of the ERK inactivation by DIF and the spontaneous feedback activation by the cells. It should be of importance to note that the levels of ERK inhibition by DIF analogs (Fig. 4D and E) correlate well with the levels of growth inhibition by the analogs (Fig. 2A). Furthermore, the MEK inhibitor, U0126, that inactivates ERK also reduced G_1 cyclins (Fig. 5). It is thus probable that DIF induces growth arrest, at least in part, via reduction of ERK activity (Fig. 8).

It is clear that the actions of DIF are different from those of other erythroid inducers in K562 cells. For example, butyrate or hydroxyurea inactivates both ERK and JNK, and activates p38 (Witt et al., 2000; Park et al., 2001). Hemin rather activates ERK, although it induces only nonterminal erythroid differentiation (Woessmann and Mivechi, 2001). Erythropoietin activates p38 and JNK but not ERK (Nagata et al., 1998). The fine relationship between erythroid differentiation and the MAPK family remains to be elucidated.

Recently, we have shown with HeLa cells and human umbilical vein endothelial cells (HUVECs) that DIF-3 degrades cyclin D_1 via activation of GSK-3β (Takahashi-Yanaga et al., 2003). However, Western analysis showed that DIF-1 did not dephosphorylate (activate) GSK-3β at Ser^9 in K562 cells (data not shown). On the other hand, it has also been shown that DIF-1 inhibits STAT3 activity involved in gastric cancer cell proliferation via the MEK–ERK-dependent pathway (Kanai et al., 2003). However, DIF-1 did not affect the phosphorylation (activity) of STAT3 in K562 cells (data not shown).

4.2. PI-3K and Akt are not involved in DIF-induced growth arrest

Akt/PKB is a serine/threonine kinase that acts downstream of the PI-3K signaling system. To date, PI-3K and Akt have been thought to play important roles in not only cell survival but also cell differentiation (Coffer et al., 1998; Vanhaesebroeck and Alessi, 2000). Because DIF has been shown to activate PI-3K and Akt in K562 cells (Kubohara and Hosaka, 1999), the involvement of the PI-3K/Akt pathway in DIF-induced events in the cells was examined (Figs. 6 and 7). Inhibition of PI-3K by wortmannin or LY294002 did not cancel DIF-induced growth arrest (Fig. 6A and B) or ERK inactivation (Fig. 7), but the drugs by themselves inhibited both cell growth (Fig. 6A and B) and ERK activity (Fig. 7B). Thus, it is probable that the basal activity of PI-3K is required for normal cell growth and that DIF-induced growth arrest may not involve the activation of PI-3K and Akt. The downstream pathway of DIF-induced Akt activation remains to be elucidated (Fig. 8).

4.3. The actions of DIF across species

DIF-1 is a differentiation-inducing factor in the original organism *D. discoideum*, and DIF-3 is a metabolite of DIF-1 (Kay et al., 1989). These molecules exhibit antiproliferative and differentiation-inducing activities in mammals (Kubohara et al., 1995a,b; Kubohara, 1997, 1999; Miwa et al., 2000). Quite recently, it was also shown that DIF-1 inhibits progesterone-induced oocyte maturation (meiosis) in *Xenopus laevis* (Kubohara et al., 2003). Since THPH, 2-MIDIF-1, or 6-MIDIF-3 exhibits much weaker antiproliferative activity (Fig. 2) (Kubohara et al., 1995a; Kubohara, 1999), the effects of DIF-1 and DIF-3 in mammalian and amphibian cells should be structure-specific.

To simply understand the multieffects of DIF beyond species, we may assume that there are counterparts of DIF in mammals (i.e., DIF-like or DIF-related molecules), the role(s) of which can be mimicked by the *Dictyostelium* factor, DIF, at relatively high concentrations. Because the soil microorganism (fungus) *Chaetomium* produces differanisole A (an analog of DIF) (Oka et al., 1985), it is probable that DIF-like molecules have similar functions as differentiation-inducing factors beyond species, at least in the lower eukaryotes.

At any rate, DIF-like molecules should be useful pharmacological tools for the analysis of mammalian cell functions, which may give rise to the development of novel drugs for cancer and some other diseases in the future.

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