Dictyostelium Differentiation-inducing Factor-3 Activates Glycogen Synthase Kinase-3β and Degrades Cyclin D1 in Mammalian Cells*

Fumi Takahashi-Yanaga‡‡, Yoji Taba‡, Yoshikazu Miwa‡, Yuzuru Kubohara‡, Yutaka Watanabe‡, Masato Hirata***, Sachio Morimoto‡, and Toshiyuki Sasaguri‡

From the ‡Department of Clinical Pharmacology, Graduate School of Medical Sciences and **Department of Molecular and Cellular Biochemistry, Graduate School of Dental Sciences, Kyushu University, Fukuoka 812-8582, Japan, †BioSignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-8512, Japan, and the ¶Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790-8577, Japan

Received for publication, June 11, 2002, and in revised form, October 30, 2002
Published, JBC Papers in Press, January 8, 2003, DOI 10.1074/jbc.M205768200

In search of chemical substances applicable for the treatment of cancer and other proliferative disorders, we studied the signal transduction of Dictyostelium differentiation-inducing factors (DIFs) in mammalian cells mainly using HeLa cells. Although DIF-1 and DIF-3 both strongly inhibited cell proliferation by inducing G0/G1 arrest, DIF-3 was more effective than DIF-1. DIF-3 suppressed cyclin D1 expression at both mRNA and protein levels, whereas the overexpression of cyclin D1 overrode DIF-3-induced cell cycle arrest. The DIF-3-induced decrease in the amount of cyclin D1 protein preceded the reduction in the level of cyclin D1 mRNA. The decrease in cyclin D1 protein seemed to be caused by accelerated proteolysis, since it was abrogated by N-acetyl-Leu-Leu-norleucinal, a proteasome inhibitor. DIF-3-induced degradation of cyclin D1 was also prevented by treatment with lithium chloride, an inhibitor of glycogen synthase kinase-3β (GSK-3β), suggesting that DIF-3 induced cyclin D1 proteolysis through the activation of GSK-3β. Indeed, DIF-3 dephosphorylated Ser32 and phosphorylated tyrosine on GSK-3β, and it stimulates GSK-3β activity in an in vitro kinase assay. Moreover, DIF-3 was revealed to induce the nuclear translocation of GSK-3β by immunofluorescent microscopy and immunoblotting of subcellular protein fractions. These results suggested that DIF-3 activates GSK-3β to accelerate the proteolysis of cyclin D1 and that this mechanism is involved in the DIF-3-induced G0/G1 arrest in mammalian cells.

Differentiation-inducing factors (DIFs)†† were identified in Dictyostelium discoideum as the morphogens required for stalk cell differentiation of Dictyostelium (1). In the DIF family, DIF-1 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone) was the first to be identified, and DIF-3, the monochlorinated analogue of DIF-1, is a natural metabolite of DIF-1 in Dictyostelium (2). However, the actions of DIFs are not limited to Dictyostelium. They also have strong effects on mammalian cells. DIF-1 and/or DIF-3 strongly inhibit proliferation and induce differentiation in several leukemia cells, such as the murine erythroleukemia cell line B8, human leukemia cell line K562, and human myeloid leukemia cell line HL-60 (3, 4). DIF-3 has been reported to have the most potent antiproliferative effect on mammalian leukemia cells among DIF analogues examined to date (5). Recently, we found that DIF-1 strongly inhibits proliferation and induces differentiation in human vascular smooth muscle cells, indicating that cells sensitive to DIFs are not limited to transformed cells (6).

However, the target molecule (receptor) of DIFs is unknown, and it is not clear even in Dictyostelium how DIFs induce an antiproliferative effect and cell differentiation. DIFs are small hydrophobic molecules and are therefore expected to be able to cross cell membranes without requiring channels or carriers. Also, the rapidity with which DIFs induce prestalk cell-specific gene expression suggests that they directly regulate gene expression. Therefore, the target molecule(s) for DIFs may be located in cytoplasm or nucleus (7). Although the precise mechanisms underlying their antiproliferative and differentiation-inducing effects are not yet known, we found that DIF-1 induces cell cycle arrest at G0/G1 phase by suppressing the expression of cyclin D1 (6). Cyclin D1 is synthesized early in G1 phase and plays a key role in the initiation and progression of this phase. When cells enter the S phase, cyclin D1 is rapidly degraded by ubiquitin-protasome-dependent proteolysis (8).

Therefore, in the present study, we investigated the mechanism underlying the DIF-induced inhibition of cyclin D1 expression. We particularly paid attention to the possible involvement of glycogen synthase kinase-3β (GSK-3β), because this serine/threonine protein kinase has been shown to regulate not only cyclin D1 gene transcription by phosphorylating β-catenin but also cyclin D1 proteolysis by directly phosphorylating cyclin D1 itself (9–12). Here we show for the first time that DIF-3, which is more effective than DIF-1 in inhibiting cell proliferation, induces the rapid degradation of cyclin D1 and the activation of GSK-3β.

MATERIALS AND METHODS

Chemicals—DIF-1 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone) was purchased from Affiniti Research Products. DIF-3 (1-(3-chloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone) was synthesized by Toyama Chemical Co. (Tokyo, Japan). U0126 was purchased from Cell Signaling Technology. N-acetyl-Leu-Leu-norleucinal and wortmannin were purchased from Sigma.

Cell Culture and Transfection—HeLa cells and bovine aortic endothelial cells (BAECs) were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 0.1 µg/ml streptomycin. The cells were plated on...
plastic tissue culture dishes or coverslips. Human umbilical vein endothelial cells were plated on 0.1% gelatin-coated dishes and maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 20% fetal bovine serum, 5 ng/ml bovine fibroblast growth factor, 100 units/ml penicillin G, and 0.1 g/ml streptomycin. Wild-type human cyclin D1 cDNA was provided by Dr. K. Tamai (Medical and Biological Laboratories Co., Nagano, Japan) and subcloned into pcDNA3 (Invitrogen). Transfection was carried out using TransIT-LT1 (Mirus), and transfected cells were maintained in growth medium for 16 h before stimulation.

**Cell Proliferation Assay**—The cells were plated on 24-well plates (0.5 × 10^4 cells/well) and treated with or without various amounts of DIF-1 or DIF-3 for given periods. Cells were harvested by the trypsin/EDTA treatment and enumerated.

**Flow Cytometry**—Cells harvested by the trypsin/EDTA treatment were suspended in hypotonic fluorochrome solution containing 50 µg/ml of propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100 (13). Cells (5 × 10^5) from each sample were analyzed for fluorescence by a Becton-Dickinson FACScalibur (Franklin Lakes, NJ).

**mRNA Expression Analysis**—Total cellular RNA was extracted with Isogen (Nippon Gene). Using 1 µg of the RNA, the expression of cyclin D1 mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using Ready-To-Go RT-PCR Beads (Amersham Biosciences) (6).

**Purification of Nucleic and Cytoplasmic Proteins**—Nucleic and cytoplasmic proteins were purified from cells cultured in 100-mm plates using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce). Five µg of each sample was subjected to Western blot analysis.

**Immunoblotting**—Samples were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a semidy transfer system (1 h, 15 V). After blocking with 5% skim milk or 5% bovine serum albumin for 1 h, the membrane was probed with a first antibody. For the polyclonal anti-cyclin D1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the polyclonal anti-cyclin D2 antibody (Santa Cruz Biotechnology), the polyclonal anti-phospho-GSK-3β (Ser9) antibody (Cell Signaling Technology), the polyclonal anti-phospho-Akt (Ser 473) antibody (Cell Signaling Technology), the polyclonal anti-phospho-p90RSK (Ser380) antibody (Cell Signaling Technology), the monoclonal anti-phosphotyrosine antibody (PY-20; Santa Cruz Biotechnology), the monoclonal anti-GSK-3β antibody (BD Transduction Laboratories), and the monoclonal anti-cyclin D3 antibody (Santa Cruz Biotechnology), the incubation was carried out overnight at 4 °C. For the monoclonal anti-phosphotyrosine antibody (PY-20, Santa Cruz Biotechnology), the monoclonal anti-GSK-3β antibody (BD Transduction Laboratories), and the monoclonal anti-cyclin D3 antibody (Santa Cruz Biotechnology), the incubation was carried out for 1 h at room temperature. The membrane was washed three times and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Bio-Rad) for 1 h. Immunoreactive proteins on the membrane were visualized by treatment with a detection reagent (LumiGLO, Cell Signaling Technology). An optical densitometric scan was performed using Science Lab 99 Image Gauge Software (Fuji Photo Film).

**Immunoprecipitation**—HeLa cells (1 × 10^6 cells) were incubated with or without DIF-3 for the periods indicated. Cells were lysed on ice for 1 h in 1 ml of the lysis buffer (50 mM NaCl, 5 mM NaF, 2 mM Na_2VO_4, 5 mM DTT, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitors) and centrifuged at 14,000 x g for 10 min at 4 °C. Immunoprecipitation was performed with polyclonal anti-cyclin D1 antibody, polyclonal anti-cyclin D2 antibody (Santa Cruz Biotechnology), or monoclonal anti-GSK-3β antibody (BD Transduction Laboratories). After washing three times with lysis buffer, the immunoprecipitates were subjected to Western blot analysis.
FIG. 2. The effect of DIF-3 on D-type cyclin expression. HeLa cells were incubated with or without DIF-3 (30 μM) for the periods indicated. A, RT-PCR analysis for cyclin D1. Total RNA (1 μg) was subjected to RT-PCR for cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR cycle numbers were 24 for cyclin D1 and 20 for glyceraldehyde-3-phosphate dehydrogenase. The result demonstrated is representative of three other experiments. B, Western blot analysis for cyclin D1. Samples were separated by 12% SDS-PAGE, and immunoblot analysis was performed using an anti-cyclin D1 antibody. Demonstrated are the results representative of three other experiments. C, Western blot analysis for cyclins D2 and D3. Samples were separated by 12% SDS-PAGE and immunoblotted using an anti-cyclin D2 antibody and an anti-cyclin D3 antibody. Demonstrated are the results representative of three other experiments.

FIG. 3. Overexpression of cyclin D1 disabled DIF-3 from inhibiting cell cycle. A, BAECs untransfected or transfected with the plasmids indicated were incubated with or without DIF-3 (30 μM) for 24 h. Protein samples were subjected to immunoblot analysis using an anti-cyclin D1 antibody. The pcDNA3/cyclin D1-transfected cells expressed an ~15-fold excessive amount of cyclin D1 compared with untransfected cells. Demonstrated are the results representative of three other experiments. B, cell cycle distribution. Untransfected and transfected BAECs were incubated with or without DIF-3 (30 μM) for 12 h. Cells were then stained with propidium iodide (PI), and the nuclear fluorescent levels were measured with a flow cytometer. The percentages of cell number in the cell cycle phases are shown as means ± S.E. of three independent experiments performed in duplicate.
FIG. 4. DIF-3 induced proteolysis of cyclin D1. A, the effect of a proteasome inhibitor. Cells pretreated with or without N-acetyl-Leu-Leu-norleucinal (20 μM) for 3 h were incubated in the presence or absence of DIF-3 (30 μM) for 3 h, and immunoblot analysis was performed using an anti-cyclin D1 antibody. The result is representative of three other experiments. B, the rate of cyclin D1 degradation. HeLa cells were incubated with or without DIF-3 (30 μM) in the presence of cycloheximide (5 μM). Cells were lysed in SDS sample buffer at the times indicated and subjected to immunoblot analysis for cyclin D1. The expression levels of cyclin D1 were quantified by densitometry and statistically analyzed. Values are means ± S.E. of three independent experiments. *, p < 0.01 compared with the value at time 0 (Student’s t test).

FIG. 5. Lithium chloride inhibited the effects of DIF-3. A, the effect of lithium chloride on DIF-3-induced cyclin D1 degradation. HeLa cells were pretreated with or without lithium chloride (20 mM) for 3 h and then incubated in the presence or absence of DIF-3 (30 μM) for 3 h. Protein samples were separated by 12% SDS-PAGE and immunoblotted with anti-GSK-3β antibody (1:200) and then incubated in the presence or absence of DIF-3 (30 μM) for 24 h. Cells were enumerated after harvested by the trypsin/EDTA treatment. Values are means ± S.E. for three independent experiments. B, the effect of lithium chloride on cell proliferation. Cells were treated with or without lithium chloride (20 mM) for 3 h and then incubated in the presence or absence of DIF-3 (30 μM) for 24 h. Cells were enumerated after harvested by the trypsin/EDTA treatment. Values are means ± S.E. for three independent experiments. C, data presented in B are differently expressed. The numbers of cells treated with DIF-3 are shown as percentages of the cell numbers obtained in the absence of DIF-3. Values are means ± S.E. for three independent experiments. *, p < 0.05; **, p < 0.001 (Student’s t test).

RESULTS

DIFs Inhibited HeLa Cell Proliferation—DIFs exhibit powerful antiproliferative effect in leukemia cells (3–5). In the present study, we first examined whether DIFs also inhibit the proliferation of HeLa cells. As shown in Fig. 1, A and B, DIF-1 and DIF-3 both strongly inhibited HeLa cell proliferation in a dose-dependent fashion, suggesting that DIFs are also effective in solid tumors. These antiproliferative effects were unlikely to be caused by cytotoxicity, because the number of dead cells indicated by the trypan blue exclusion test was not increased by the treatment with DIFs (data not shown). Consistent with the result obtained in leukemia cells (5), DIF-3 was more effective than DIF-1 in HeLa cells. Therefore, we used DIF-3 in the subsequent experiments. We next examined the cell cycle distribution using flow cytometry. Although the cell populations in S and G2/M phases decreased after the treatment with DIF-3, the population in G1 phase significantly increased.
Fig. 6. DIF-3 activates GSK-3β.

HeLa cells were incubated with or without DIF-3 (30 μM) for the periods indicated. A, in vitro kinase assay. GSK-3β was immunoprecipitated from cell lysates and measured for kinase activity using a substrate peptide derived from the sequence of glycogen synthase. The results are means ± S.E. of three independent experiments performed in duplicate. *, p < 0.01 compared with the control at time 0 (Student's t test). B, the effect of lithium chloride on GSK-3β activity. GSK-3β immunoprecipitated from cell lysates was measured for kinase activity using a substrate peptide derived from the sequence of glycogen synthase in the presence or absence of 10 mM lithium chloride. The results are means ± S.E. of three independent experiments. C, phosphorylation of Ser9 on GSK-3β. Cell lysates were subjected to immunoblot analysis using an anti-phospho-GSK-3β (Ser9) antibody. The levels of Ser9 phosphorylation on GSK-3β were quantified by densitometry and shown as percentages of the levels in the control cells. Values are means ± S.E. of three independent experiments. *, p < 0.01 compared with the control (Student's t test). D, tyrosine phosphorylation of GSK-3β. Immunoprecipitated GSK-3β was subjected to immunoblot analysis using an anti-phosphotyrosine antibody (PY-20). The membrane was reprobed with an anti-GSK-3β antibody. The levels of tyrosine phosphorylated GSK-3β were quantified and shown as percentages of the levels in the control cells. Values are means ± S.E. of three independent experiments. *, p < 0.01 compared with the control (Student's t test).

DIF-3 Activates GSK-3β

DIF-3-induced G0/G1 Arrest Was Rescued by the Overexpression of Cyclin D1—To clarify whether the overexpression of cyclin D1 is able to rescue cells from cell cycle arrest induced by DIF-3, wild-type human cyclin D1 cDNA was transfected to BAECs, since transfection efficiency was the highest in BAECs among mammalian cell species we examined including HeLa cells. As shown in Fig. 3A, the expression levels of cyclin D1 in untransfected cells and in cells transfected with empty pcDNA3 were reduced after 24 h incubation with DIF-3, demonstrating that DIF-3 showed the same effect in BAECs as in HeLa cells. However, the expression level of cyclin D1 in cells transfected with pcDNA3/cyclin D1 was not significantly changed by DIF-3 treatment. We then examined the cell cycle distribution using untransfected and transfected cells. Although DIF-3 induced G0/G1 arrest in untransfected and pcDNA3-transfected cells, there was no significant difference in pcDNA3/cyclin D1-transfected cells between the absence and presence of DIF-3 treatment (Fig. 3B). Therefore, DIF-3 was likely to induce cell cycle arrest by reducing the expression level of cyclin D1.

DIF-3 Suppressed the Expression of Cyclins D1, D2, and D3 in HeLa Cells—We reported that DIF-1 induced G0/G1 arrest, suppressing the expression of cyclins D1, D2, and D3 in human vascular smooth muscle cells (6). In HeLa cells, DIF-3 also reduced both the mRNA and protein levels of cyclin D1 (Fig. 2, A and B). RT-PCR analyses showed that the cyclin D1 mRNA level was not significantly affected after a 1-h incubation with DIF-3 (Fig. 2A). Although it began to slowly decrease from 3 h, a considerable amount of cyclin D1 mRNA was still expressed until 6 h. Despite this slow decrease in the level of mRNA, DIF-3 rapidly reduced the protein level of cyclin D1 (Fig. 2B). Cyclin D1 protein markedly decreased after 1 h of incubation with DIF-3 and nearly completely disappeared by 3 h. This rapid decrease in the amount of protein was not explained by suppression of mRNA expression. The protein levels of cyclins D2 and D3 also significantly decreased after 1 h of treatment with DIF-3 and almost disappeared after 6 h of treatment (Fig. 2C).

(Fig. 1C), indicating that DIF-3 induced G0/G1 arrest in HeLa cells. This result was consistent with our previous study as to the effect of DIF-1 on vascular smooth muscle cell cycle (6).
DIF-3 Activates GSK-3β—The activity of GSK-3β was measured using an in vitro kinase assay. DIF-3 (30 μM) elevated GSK-3β activity by 1.9-fold after 30 min incubation, and GSK-3β was still activated at 3 h (Fig. 6A). In the presence of lithium chloride, however, DIF-3 was not able to activate GSK-3β (Fig. 6B). Since GSK-3β is activated by the dephosphorylation of Ser9 (9–11), the level of GSK-3β Ser9 phosphorylation was examined using an anti-phospho-GSK-3β (Ser9) antibody. As shown in Fig. 6C, DIF-3 dramatically reduced the phosphorylation level of Ser9 on GSK-3β after the incubation with DIF-3 for 30 min, and the phosphorylation level of Ser9 was slowly recovered, looking like a mirror image of the time course of GSK-3β activity (Fig. 6A). Further, we examined the tyrosine phosphorylation level of GSK-3β since the activity of this enzyme has been reported to be increased by phosphorylation of Tyr216 (9–11). As shown in Fig. 6D, DIF-3 significantly elevated the tyrosine phosphorylation level of GSK-3β by 2.0-fold after the incubation with DIF-3 for 30 min. These results strongly indicated that DIF-3 activates GSK-3β.

DIF-3 Induced Nuclear Translocation of GSK-3β—GSK-3β is a cytosolic protein; however, it is translocated into the nucleus when activated (9–11). GSK-3β thereby accumulated in the nucleus phosphorylates cyclin D1 and excludes it from nucleus, resulting in its degradation in the cytoplasm (12). To test whether DIF-3-activated GSK-3β is able to target cyclin D1, we examined the subcellular distribution of GSK-3β after stimulation with DIF-3. Immunofluorescent staining for GSK-3β revealed that GSK-3β was most present in the cytoplasm and that there was only a small amount in nuclei in unstimulated cells; however, it was markedly translocated into nuclei after stimulation with DIF-3 (Fig. 7A). Importantly, the time course of GSK-3β translocation into nuclei was similar to that of cyclin

**FIG. 7. DIF-3 induces GSK-3β nuclear accumulation.** A and B. Immunofluorescent staining with a monoclonal (A) or a polyclonal (B) anti-GSK-3β antibody. The results are representative of three other experiments. C. Western blot analysis. Nuclear and cytoplasmic proteins were purified from HeLa cells incubated with or without DIF-3 (30 μM) for 2 h. Western blot analysis was carried out using a monoclonal anti-GSK-3β antibody. The results are representative of three other experiments.
DIF-3 Activates GSK-3β

In the present study, we showed that DIF-3 accelerated the degradation of cyclin D1 by activating GSK-3β mainly using HeLa cells. However, this effect of DIF-3 was also found in other cell species including transformed and normal (untransformed) cells. As an example, we have demonstrated the results obtained in vascular endothelial cells. Therefore, DIF-3 may ubiquitously activate GSK-3β (Fig. 9C), indicating that these effects of DIF-3 are not limited to transformed cells but common between transformed and untransformed cells.

**DISCUSSION**

In the present study, we showed that DIF-3 accelerated the degradation of cyclin D1 by activating GSK-3β mainly using HeLa cells. However, this effect of DIF-3 was also found in other cell species including transformed and normal (untransformed) cells. As an example, we have demonstrated the results obtained in vascular endothelial cells. Therefore, DIF-3 may ubiquitously activate GSK-3β in the wide variety of cell types. Since HeLa cells express human papilloma virus antigens E6 and E7, which inactivate retinoblastoma protein (8), it is not clear whether cyclin D1 is required for their cell cycle progres-
DIF-3 Activates GSK-3β

DIF-3, naturally generated from DIF-1 as its first metabolite, is much weaker than DIF-1 in the ability to induce stalk cell differentiation in Dictyostelium (2). However, in contrast, the antiproliferative effect of DIF-3 was significantly stronger than that of DIF-1 in HeLa cells, consistent with a previous report that DIF-3 is more effective than DIF-1 at inhibiting proliferation in K562 human leukemia cells (5). This species difference in the sensitivity to DIFs may be caused by a difference in the nature of the target molecule (such as the affinity for DIFs) between mammalian and Dictyostelium cells.

We found that DIF-3 not only elevated the activity of GSK-3β but also induced nuclear translocation of the kinase. GSK-3β was initially considered to be a soluble protein expressed in cytoplasm; however, GSK-3β would have no access to nuclear proteins such as cyclin D1 if it resided in cytoplasm. Recent evidence has indicated that GSK-3β in nucleic phosphorylates nuclear proteins, such as cyclin D1 (12), nuclear factor of activated T-cells (17), heat shock factor-1 (18), and cAMP-response element-binding protein (19). Indeed, GSK-3β has been identified from the nuclei of cell-cycle-arrested NIH-3T3 cells (12), cardiomyocytes stimulated with endothelin-1 (20), and heat shock- and staurosporine-treated SH-SY5Y human neuroblastoma cells (21). Therefore, DIF-3 seems to make it possible for GSK-3β to phosphorylate cyclin D1 by translocating GSK-3β into the nucleus.

Not only a rapid proteolysis but also a reduction in cyclin D1 mRNA expression was induced by DIF-3, although this latter effect took much longer time. Cyclin D1 gene expression is activated by β-catenin, the degradation of which is also initiated by GSK-3β (22). Therefore, activation of GSK-3β was expected to lead to a reduction in both protein and mRNA levels of cyclin D1 through independent pathways. Our results agreed well with this rationale. The proteolysis and mRNA reduction both seemed to be explained by DIF-3-induced GSK-3β activation.

The target molecule for DIFs is still unknown even in Dictyostelium. Since DIF-3 activates GSK-3β, a target for DIF-3 may be a protein closely related to the regulation of GSK-3β activity. The activity of GSK-3β is decreased by phosphorylation of Ser9 and increased by phosphorylation of Tyr216 on GSK-3β (23). Akt activated by PI3K and p90Rsk activated by the MAPK cascade are the candidate molecules to modulate GSK-3β activity by Ser9 phosphorylation (16). Although DIF-3 did not affect the Ser9 phosphorylation of Akt, it strongly induced phosphorylation on Ser9 on p90Rsk. However, we were not able to elucidate the role of DIF-3-induced p90Rsk activation, since this kinase did not seem to be involved in cyclin D1 degradation induced by DIF-3 (Fig. 6B). DIF-3 also enhanced tyrosine phosphorylation on GSK-3β. Recently, a novel non-receptor tyrosine kinase, ZAK-1, has been found to directly activate GSK-3β in Dictyostelium (24). However, a ZAK-1 counterpart in mammals has not yet been discovered. A target molecule for DIF-3 might be a novel protein kinase or phosphatase controlling GSK-3β activity in mammalian cells. Recently, it has been reported that not only phosphorylation but also distribution regulates GSK-3β activity (25). Thus, DIF-3-induced accumulation of GSK-3β in nuclei might have an important role to regulate GSK-3β activity. In addition, mammalian cells have a target molecule for DIFs as Dictyostelium, one could hypothesize that mammals also produce DIF-like substances to control growth and differentiation.

The Wnt signaling pathway is essential for embryonic development, cell proliferation, cell differentiation, microtubule dynamics, and cell motility. Several mutations have been identified in the components of the Wnt pathway in a variety of malignant tumors. For instance, most human colon cancers have mutations in the APC gene that result in the accumulation of β-catenin (26). Mutations in β-catenin, which amplify the accumulation of β-catenin itself, also have been identified in colon cancers (27), malignant melanomas (26), prostate cancers (27), and hepatocellular carcinomas (28). β-Catenin accumulated to an abnormal level would produce an excessive amount of cyclin D1 mRNA and promote tumor growth (29). Therefore, chemists that activate GSK-3β, such as DIFs, may be useful for the treatment of several cancers.

Recently, it has been reported that proapoptotic stimuli, such as heat shock and staurosporine, activate GSK-3β and induce its accumulation in nucleus (21). However, cytotoxic or proapoptotic agents damage not only cancer cells but also normal cells, which would cause severe adverse drug events. Distinct from staurosporine and other proapoptotic agents, DIF-3 did not induce the activation of caspase-3 (data not shown). Therefore, the antiproliferative effect of DIF-3 did not seem to be caused by the induction of apoptosis. DIF-3 is a unique compound that activates GSK-3β but does not induce apoptotic cell death.

Acknowledgments—We thank Katsuyuki Tamai (Medical and Biological Laboratories Co., Nagano, Japan) for kindly providing human cyclin D1 cDNA, Hiroki Yoshida (Medical Institute of Bioregulation, Kyusyu University) for technical advice, and Toyama Chemical Co. for synthesizing DIF-3.

REFERENCES
1. Morris, H. R., Taylor, G. W., Maseno, M. S., Jermyn, K. A., and Kay, R. R. (1987) Nature 328, 811–814.
2. Morris, H. R., Maseno, M. S., Taylor, G. W., Jermyn, K. A., and Kay, R. R. (1988) Biochem. J. 249, 903–906.
3. Ashali, K., Sakurai, A., Takahashi N., Kubohara, Y., Okamoto, K., and Tanaka, Y. (1995) Biochem. Biophys. Res. Commun. 206, 1036–1039.
4. Kubohara, Y. (1997) Biochem. Biophys. Res. Commun. 236, 418–422.
5. Kubohara, Y. (1999) Eur. J. Pharmacol. 381, 57–62.
6. Miwa, Y., Sasaguri, T., Kasuka, C., Inoue, M., Hishida, A., Abuminya, T., and Kubohara, Y. (2000) Circ. Res. 86, 68–75.
7. Insall, R., and Kay, R. R. (1999) EMBO J. 9, 3323–3328.
8. Weinberg, R. A. (1995) Cell 81, 323–330.
9. Perkey, D. M., and Kimelman, D. (2000) Dev. Biol. 235, 471–479.
10. Kim, L., and Kimmel, A. R. (2000)Curr. Opin. Genet. Dev. 10, 508–514.
11. Harwood, A. J. (2001)J. Cell 105, 821–824.
12. Dohi, J. A., Cheng, M., Rouss, M. P., and Sherr, C. J. (1998) Genes Dev. 12, 3499–3511.
13. Yoshida, H., Sumichika, H., Hamano, S., He, X., Minamishima, Y., Kimura, G., and Nomoto, K. (1995–1996)J. Virol. 69, 4769–4775.
14. Spinella, M. J., Freemantle, S. J., Sekula, D., Chang, J. H., Christie, A. J., and Ovsenek, N. (2000)Science 291, 1934–1937.
Dictyostelium Differentiation-inducing Factor-3 Activates Glycogen Synthase Kinase-3 β and Degrades Cyclin D1 in Mammalian Cells

Fumi Takahashi-Yanaga, Yoji Taba, Yoshikazu Miwa, Yuzuru Kubohara, Yutaka Watanabe, Masato Hirata, Sachio Morimoto and Toshiyuki Sasaguri

J. Biol. Chem. 2003, 278:9663-9670.
doi: 10.1074/jbc.M205768200 originally published online January 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M205768200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 14 of which can be accessed free at http://www.jbc.org/content/278/11/9663.full.html#ref-list-1