Involvement of GSK-3β and DYRK1B in Differentiation-inducing Factor-3-induced Phosphorylation of Cyclin D1 in HeLa Cells*

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Differentiation-inducing factors (DIFs) are putative morphogens that induce cell differentiation in Dictyostelium discoideum. We previously reported that DIF-3 activates glycogen synthase kinase-3β (GSK-3β), resulting in the degradation of cyclin D1 in HeLa cells. In this study, we investigated the effect of DIF-3 on cyclin D1 mutants (R29Q, L32A, T286A, T288A, and T286A/T288A) to clarify the precise mechanisms by which DIF-3 degrades cyclin D1 in HeLa cells. We revealed that T286A, T288A, and T286A/T288A mutants were resistant to DIF-3-induced degradation compared with wild-type cyclin D1, indicating that the phosphorylation of Thr286 and Thr288 were critical for cyclin D1 degradation induced by DIF-3. Indeed, DIF-3 markedly elevated the phosphorylation level of cyclin D1, and mutations introduced to Thr286 and/or Thr288 prevented the phosphorylation induced by DIF-3. Depletion of endogenous GSK-3β and dual-specificity tyrosine phosphorylation regulated kinase 1B (DYRK1B) by RNA interference attenuated the DIF-3-induced cyclin D1 phosphorylation and degradation. The effect of DIF-3 on DYRK1B activity was examined and we found that DIF-3 also activated this kinase. Further, we found that not only GSK-3β but also DYRK1B modulates cyclin D1 subcellular localization by the phosphorylation of Thr288. These results suggest that DIF-3 induces degradation of cyclin D1 through the GSK-3β- and DYRK1B-mediated threonine phosphorylation in HeLa cells.

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‡ The abbreviations used are: DIF, differentiation-inducing factor; GSK-3β, glycogen synthase kinase-3β; DYRK1B, dual-specificity tyrosine phosphorylation regulated kinase 1B; PDE1, calmodulin-dependent cyclic nucleotide phosphodiesterase; ASSLN, N-acetyl- Leu-Leu-Norleucinal; MKK3, mitogen-activated protein kinase kinase 3; RNAi, RNA interference; IKKα, IκB kinase α; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein.
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by DIF-3 in HeLa cells. Moreover, we revealed that not only GSK-3β but also DYRK1B was involved in the phosphorylation of cyclin D1 induced by DIF-3 in HeLa cells. Thus DIF-3 efficiently induced degradation of cyclin D1.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—DIF-3 (1-(3-chloro-2, 6-di-hydroxy-4-methoxyphenyl)-1-hexanone) was synthesized according to Masento et al. (21). SB216763 was purchased from BIOMOL international. The monoclonal anti-β-actin antibody, anti-FLAG M2 antibody, and N-acetyl-Leu-Leu-norleucinal (ALLN) were from Sigma. Polyclonal anti-cyclin D1 antibody was from Santa Cruz Biotechnology. Monoclonal anti-GAPDH antibody was from Abcam. Polyclonal anti-DYRK1B antibody was from ABGENT. Monoclonal anti-histone H3 antibody was from Upstate Biotechnology.

**Cell Culture and Transfection**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. The wild-type human cyclin D1 cDNA was subcloned into pcDNA3 (Invitrogen) as described previously (6). Human cyclin D1 mutants R29Q, L32A, T286A, T286A/T288A, and T286A/T288 were generated by a QuikChange® Site-directed Mutagenesis kit (Stratagene). The FLAG-tagged human cyclin D1 constructs were also generated. Human cyclin D1 pGL3 basic luciferase reporter construct was a generous gift from Drs. O. Tetsu and F. McCormick, University of California San Francisco. Transfection was carried out using Lipofectamine™ Plus transfection reagent (Invitrogen).

**RNA Interference (RNAi)**—GSK-3β validated Stealth™ RNAi was purchased from Invitrogen. Double-stranded Stealth™ RNAi specifically targeting human DYRK1B (5’-gc-gcgguaauagacugucucua-3’) was synthesized (Invitrogen). Transfection of RNAi was carried out according to the manufacturer protocol using Lipofectamine™ 2000 transfection reagent (Invitrogen). Stealth™ RNAi negative controls, which are the GC-matched scrambled sequence, were also purchased from Invitrogen.

**Western Blot Analysis**—Samples were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a semi-dry transfer system (1 h, 12 V). After blocking with 5% skim milk for 1 h, the membrane was probed with a first antibody. The membrane was washed three times and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology) for 1 h. Immuno-reactive 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).

**Luciferase Reporter Assay**—HeLa cells were transfected with luciferase reporter plasmid and pRL-SV40, a Renilla luciferase expression plasmid (Toyo Ink Mfg Co) for the control of transfection efficiency. Cells were cultured for 24 h after transfection and stimulated with DIF-1 (30 μM) for 1 h. The luciferase activity was determined with a luminometer (Lumat LB 9507, Berthold Technologies) and normalized with respect to Renilla luciferase activity.

**In Vitro DYRK1B Kinase Reaction**—The in vitro kinase assay of DYRK1B was carried out according to Lim et al. (22). Briefly, cells were stimulated with or without DIF-3 and immunoprecipitation was carried out using 2 μg of anti-DYRK1B antibody. The immunoprecipitated samples were washed twice with lysis buffer and twice with a kinase assay buffer (20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, and 1 mM dithiothreitol). The kinase activities of DYRK1B were tested with 30 μM of kinase assay buffer con-
taining 20 mM Tris/HCl (pH 7.4), 5 mM MgCl₂, 1 mM dithio- 
reitol, 250 μM ATP, 5 μCi of [γ-32P]ATP (Amersham Bio-
sciences), and 20 μg of myelin basic protein (MBP) from Upstate Biotechnology. The samples were incubated at 30 °C 
for 30 min, and the reaction was terminated by adding 10 μl of 
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FIGURE 2. DIF-3 induced phosphorylation on cyclin D1. A, effect of DIF-1 on the time course of cyclin D1 phosphorylation. Cells were metabolically labeled with [32P]orthophosphate in the presence of ALLN (20 μM) and stimulated with DIF-3 (30 μM) for the indicated periods. Samples were subjected to immunoprecipitation using anti-cyclin D1 antibody. After electrophoresis and transfer, phosphorylated-cyclin D1 was visualized by autoradiography. The membrane was then subjected to Western blot analysis for cyclin D1. The levels of phosphorylated-cyclin D1 (p-cyclin D1) to cyclin D1 amount (total cyclin D1). Values are means ± S.E. of three independent experiments. *, p < 0.01, compared with the control (Student’s t test). B, effect of LiCl. Cells were metabolically labeled with [32P]orthophosphate in the presence of ALLN (20 μM) and LiCl (30 mM), followed by the stimulation with DIF-3 (30 μM) for 1 h. Values are means ± S.E. of three independent experiments. *, p < 0.01 compared with columns indicated (t test). C, effect of Thr286 and/or Thr288 mutation on cyclin D1 phosphorylation induced by DIF-3. Cells were transfected with the plasmid containing indicated type of cyclin D1. Transfected cells were metabolically labeled with [32P]orthophosphate in the presence of ALLN (20 μM), followed by the stimulation with DIF-3 (30 μM) for 1 h. Values are means ± S.E. of three independent experiments. *, p < 0.01, compared with columns indicated (Student’s t test).

Effect of DIF-3 on Cyclin D1 and Its Mutants—There are at least three independent motifs in cyclin D1 involved in its degradation. The first one is the Arg-X-X-Leu destruction box (Arg29-X-X-Leu32), which plays a major role in rendering cyclin D1 susceptible to degrada-
tion by ionizing radiation (11). The second one is Thr286, which is phosphorylated by GSK-3β to induce cyclin D1 degradation (12, 13). The third one is Thr288, recently identified by Zou et al. (14), who reported that a serine/threonine kinase DYRK1B phosphorylates cyclin D1 at Thr288, also leading to the degradation of cyclin D1. Therefore, we investigated five different mutants of cyclin D1 (R29Q, L32R, T286A, T288A, and T286A/T288A) to determine the mechanisms by which DIF-3 induces cyclin D1 degradation. As shown in Fig. 1A, the effect of DIF-3 on overexpressed wild-type cyclin D1 was similar to the effect on intrinsic cyclin D1. While R29Q and L32R mutants were fully responsive to DIF-3, T286A, T288A, and T286A/T288A mutations significantly reduced the effect of DIF-3 (Fig. 1B). Among these three mutations, T286A/T288A most strongly resisted DIF-3. These results suggest that the phosphorylation of both Thr286 and Thr288 are critical for DIF-3-induced cyclin D1 degradation. To analyze the effect of DIF-3 on cyclin D1 promoter activity, we performed luciferase reporter assay using the human cyclin D1 promoter construct pGL3-basic vector (8). However, DIF-3 did not have significant effect on promoter activity after 1 h incubation (440757.8 ±
Thr<sup>286</sup> residues are involved in DIF-3-induced cyclin D1 phosphorylation, mutated cyclin D1 proteins (T286A, T288A and T286A/T288A) were overexpressed in HeLa cells. The effect of DIF-1 was greatly attenuated in the T286A mutant and significantly reduced in the T288A cyclin D1 mutant (Fig. 2C). T286A/T288A double mutation almost completely abolished the phosphorylation induced by DIF-3. This result was well correlated with the result shown in Fig. 1B. Thus, DIF-3 seemed to induce cyclin D1 phosphorylation at Thr<sup>286</sup> and Thr<sup>288</sup> triggering cyclin D1 degradation.

**Inhibition of GSK-3β Attenuated DIF-3-induced Cyclin D1 Degradation**—We previously reported that DIF-3 activated GSK-3β and induced cyclin D1 degradation by the acceleration of ubiquitin-proteasome-dependent proteolysis in HeLa cells (6). To elucidate the role of GSK-3β in DIF-3-induced cyclin D1 degradation, HeLa cells were pretreated with SB216763, a specific GSK-3α and β inhibitor. As shown in Fig. 3A, although SB216763 (20 μM) attenuated the degradation of cyclin D1 induced by DIF-3, this compound was shown not fully inhibit DIF-3 action, even if this concentration of SB216763 caused complete inhibition of GSK-3β activity in *in vitro kinase assay* using phospho-Glycogen Synthase Peptide-2 (Upstate Biotechnology) as substrate (data not shown). Subsequently, we attempted to deplete endogenous GSK-3β using RNAi to determine the involvement of GSK-3β in DIF-3 action. As shown in Fig. 3B, the protein level of GSK-3β was markedly reduced by transfection with GSK-3β RNAi. The depletion of GSK-3β by transfection with GSK-3β RNAi attenuated the effect of DIF-3 on cyclin D1 degradation, while control RNAi (GC-matched scrambled sequence) did not have a significant effect (Fig. 3C).

These results clearly indicated the involvement of GSK-3β in the DIF-3-induced cyclin D1 degradation but GSK-3β was unlikely to be the only kinase responsible for cyclin D1 degradation induced by DIF-3.

**Depletion of DYRK1B Attenuated the Cyclin D1 Degradation Induced by DIF-3**—Recently, it has been reported that DYRK1B phosphorylates cyclin D1 at Thr<sup>286</sup> leading to the proteolysis of cyclin D1 (14). Because DIF-3 induced phosphorylation of Thr<sup>288</sup>, the involvement of DYRK1B in the DIF-3-induced degradation of cyclin D1 was investigated. For this purpose, endogenous DYRK1B was depleted with RNAi. As shown in Fig. 4A, the protein level of DYRK1B was markedly reduced by trans-
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Depletion of DYRK1B prevented the degradation of cyclin D1 induced by DIF-3. A, depletion of DYRK1B by RNAi. Cells were transfected with control RNAi or RNAi to DYRK1B (100 nM). Protein samples were subjected to immunoblot analysis using the anti-DYRK1B antibody. The expression levels of DYRK1B were quantified by densitometry and normalized to those of GAPDH. The results are shown as percentages of cyclin D1 amounts in DIF-3-treated cells to those of GAPDH. Values are means ± S.E. of three independent experiments. *, p < 0.05 compared with the minus RNAi (Student’s t test).

B, depletion of DYRK1B by RNAi attenuated the effect of DIF-1. Cells were transfected with control RNAi or RNAi to DYRK1B (100 nM) and were incubated with or without DIF-3 (30 μM) for 1 h. Protein samples were subjected to immunoblot analysis using the anti-cyclin D1 antibody. The expression levels of cyclin D1 were quantified by densitometry and normalized to those of GAPDH. The results are shown as percentages of cyclin D1 amounts in DIF-3-treated cells to those in control cells. Values are means ± S.E. of three independent experiments. *, p < 0.05 compared with the minus RNAi (Student’s t test).

DYRK1B was activated by DIF-3. A, DIF-3 activated DYRK1B. Cells were pretreated with or without LiCl (30 mM) and stimulated with DIF-3 (30 μM) for 30 min. DYRK1B was immunoprecipitated and subjected to in vitro kinase assay using MBP as substrate in the presence or absence of LiCl. The levels of radioactive bands were quantified by densitometry and normalized to the levels of MBP. Values are means ± S.E. of three independent experiments. *, p < 0.01, compared with the columns indicated (Student’s t test). B, inhibition of DYRK1B activity by RNAi. Cells were transfected with RNAi to DYRK1B (100 nM) and stimulated with DIF-3 (30 μM) for 30 min. DYRK1B was immunoprecipitated and subjected to in vitro kinase assay. Values are means ± S.E. of three independent experiments. *, p < 0.01, compared with the columns indicated (Student’s t test).

DIF-3 Activated DYRK1B—To clarify the effect of DIF-3 on DYRK1B activity, in vitro kinase assay was carried out using MBP as substrate (22). HeLa cells were stimulated with or without DIF-3 for 30 min and DYRK1B was immunoprecipitated to subject to kinase assay. As shown in Fig. 5A, DIF-3 significantly activated DYRK1B by 200% of control after 30 min incubation. Since LiCl almost completely inhibited cyclin D1 phosphorylation induced by DIF-3, the effect of LiCl on DYRK1B activity was examined. Interestingly, LiCl also attenuated the DYRK1B activity but failed to complete inhibition of this kinase (Fig. 5A). This result was agreeable with previous report which observed that LiCl strongly inhibit GSK-3β and weakly but significantly attenuated DYRK1B (14). We subsequently examined the effect of DYRK1B RNAi. As shown in Fig. 5B, DYRK1B RNAi markedly reduced the kinase activity to 30% of control and this reduction was well correlated with the reduction of DYRK1B protein level by RNAi shown in Fig. 4A.

GSK-3β and DYRK1B Phosphorylated Thr286 and Thr288 on Cyclin D1, Respectively, and Modified Cyclin D1 Subcellular Localization—Next, we examined the effect of depletion of GSK-3β and DYRK1B on cyclin D1 phosphorylation induced by DIF-3. As shown in Fig. 6A, the depletion of GSK-3β or DYRK1B significantly reduced but did not abolish the cyclin D1 phosphorylation induced by DIF-3. Subsequently, we examined the effect of depletion of GSK-3β or DYRK1B on phosphorylation of cyclin D1 mutants to clarify which site(s) on cyclin D1, Thr286 or Thr288, are phosphorylated by GSK-3β and DYRK1B. For this experiment, we used FLAG-tagged wild-type and mutated cyclin D1 expression vectors. Fig. 6B showed that DIF-3 failed to induce phosphorylation on T288A cyclin D1 mutant after GSK-3β depletion. On the other hand, T286A cyclin D1 mutant was not significantly phosphorylated by DIF-3 treatment after depletion of DYRK1B. These results suggested that Thr286 was phosphorylated by GSK-3β and Thr288 was phosphorylated by DYRK1B, similarly to the previous reports (13, 14). We further examined the subcellular localization of wild-type and mutant cyclin D1. As shown in Fig. 6C and D, FLAG-tagged T286A and T288A cyclin D1 mutants were abundant in nucleus compared with wild-type. DIF-3 fusion with DYRK1B RNAi. Although the control RNAi (GC matched scrambled sequence) did not have a significant effect, the depletion of DYRK1B significantly attenuated the effect of DIF-1 on cyclin D1 degradation (Fig. 4B), suggesting the involvement of DYRK1B in cyclin D1 degradation induced by DIF-3.
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A

autorad

WB: cyclin D1

DIF-3 (30 μM) RNAi

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B

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WB: FLAG

DIF-3 (30 μM)

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C

wild-type

control

DIF-3

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T286A

T288A

D

Nuclear Fraction

FLAG

histone

DIF-3 (30 μM)

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% of control

wild-type T286A T288A
**FIGURE 6.** **DYRK1B and GSK-3β phosphorylated Thr286 and Thr288, respectively, and modulated cyclin D1 localization.** A, depletion of GSK-3β and DYRK1B attenuated the phosphorylation of cyclin D1 induced by DIF-3. Cells were transfected with RNAi to GSK-3β or RNAi to DYRK1B (100 nM) and metabolically labeled with [32P]orthophosphate in the presence of ALLN (20 μM), followed by stimulation with DIF-3 (30 μM) for 1 h. The levels of radioactive bands and cyclin D1 amounts were quantified and the results are shown as ratios of phosphorylated cyclin D1 (p-cyclin D1) to cyclin D1 amount (total cyclin D1). Values are means ± S.E. of three independent experiments. *, p < 0.01, compared with columns indicated (Student’s t test). B, cells were transfected with the plasmid containing indicated type of FLAG-tagged cyclin D1 and metabolically labeled with [32P]orthophosphate in the presence of ALLN (20 μM), followed by the stimulation with DIF-3 (30 μM) for 1 h. Values are means ± S.E. of three independent experiments. *, p < 0.01, compared with control (Student’s t test). C, immunofluorescence. Cells were plated on coverslips and transfected with the plasmid containing indicated type of FLAG-tagged cyclin D1. After a 3-h incubation with or without DIF-3 (30 μM), immunofluorescent staining was performed with the monoclonal anti-FLAG antibody. The expression levels of FLAG-tagged cyclin D1 were quantified by densitometry and normalized to those of histone H3. The results are shown as percentages of cyclin D1 from the nucleus, whereas T286A and T288A mutations significantly reduced the effect of DIF-3, suggesting that not only Thr286 but also Thr288 plays an important role to modulate the subcellular localization of cyclin D1. Taken together, Thr286 and Thr288 were likely to be phosphorylated by GSK-3β and DYRK1B, respectively, and subcellular localization of cyclin D1 seemed to be modulated by GSK-3β and DYRK1B.

**FIGURE 7.** **GSK-3β and DYRK1B were independently involved in cyclin D1 degradation induced by DIF-3.** A, GSK-3β and DYRK1B worked independently. Cells were transfected with RNAi to GSK-3β and/or RNAi to DYRK1B (100 nM) and metabolically labeled with [32P]orthophosphate in the presence of ALLN (20 μM) for 1 h. Protein samples were subjected to immunoblot analysis using the anti-cyclin D1 antibody. The expression levels of cyclin D1 were quantified by densitometry and normalized to those of GAPDH. The results are shown as percentages of cyclin D1 amounts in wild-type cyclin D1-transfected cells. Values are means ± S.E. of three independent experiments. *, p < 0.05 compared with columns indicated (Student’s t test). B, cell number in the cell cycle phases are shown in as means ± S.E. of three independent experiments performed in triplicate. *, p < 0.01 compared with DIF-3-treated control (Student’s t test).

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induced export of wild-type cyclin D1 from the nucleus, whereas T286A and T288A mutations significantly reduced the effect of DIF-3, suggesting that not only Thr286 but also Thr288 plays an important role to modulate the subcellular localization of cyclin D1. Taken together, Thr286 and Thr288 were likely to be phosphorylated by GSK-3β and DYRK1B, respectively, and subcellular localization of cyclin D1 seemed to be modulated by GSK-3β and DYRK1B.

GSK-3β and DYRK1B Were Independently Involved in Cyclin D1 Degradation Induced by DIF-3—Furthermore, to investigate the relationship between GSK-3β and DYRK1B in DIF-3 action, RNAi of GSK-3β and DYRK1B were co-transfected. As shown in Fig. 7A, co-transfection of GSK-3β and DYRK1B RNAs exhibited an additive effect for cyclin D1 degradation, suggesting that GSK-3β and DYRK1B independently induce the phosphorylation and the degradation of cyclin D1. Because we previously reported that cyclin D1 depletion is associated with cell cycle arrest following exposure to DIF-3 (6), we examined the effects of reduction of GSK-3β and/or DYRK1B on DIF-3-induced cell cycle arrest. Although transfection of GSK-3β or DYRK1B RNAi did not have a significant effect on the DIF-3-induced increase in the number of G0/G1 cells, co-transfection of GSK-3β and DYRK1B RNAs significantly attenuated the effect of DIF-3 (Fig. 7B). This result indicated that GSK-3β and DYRK1B played important roles in cell cycle arrest induced by DIF-3 in HeLa cells.
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DISCUSSION

Cyclin D1 degradation is facilitated by the phosphorylation of the specific threonine residues 286 and 288, according to previous reports (12–14). In this study, we found that both residues play important roles in DIF-3-induced cyclin D1 degradation.

It has been reported that GSK-3β and IkB kinase α (IKKα) phosphorylate Thr286 (23) and that DYRK1B phosphorylates Thr288 of cyclin D1. Although we examined the effect of DIF-3 on IKKα, DIF-3 did not activate this kinase (data not shown). Therefore, GSK-3β seemed to be the only candidate kinase that phosphorylated cyclin D1 at Thr286 upon DIF-3 stimulation. The activity of GSK-3β is increased by the dephosphorylation of Ser9 (15–17) and we previously reported that DIF-3 induced the dephosphorylation of Ser9 and stimulated GSK-3β activity (6). Akt and p90RSK, which are activated by phosphatidylinositol 3-kinase (PI3K) and MAPK cascade, respectively, are candidate enzymes for the phosphorylation of GSK-3β at Ser9 (24, 25). However, as we reported previously, DIF-3 did not suppress Akt and even activated p90RSK (6). MKK3 has been reported to enhance DYRK1B kinase activity (22) and we found that DIF-3 also activated MKK3. This result might indicate that DIF-3 activates DYRK1B through MKK3 activation. However, we could not understand the mechanisms how DIF-3 activates GSK-3β, DYRK1B, and/or MKK3 at present. Further study is required to clarify this point.

Shimizu et al. (4) reported that PDE1 is a pharmacological target molecule for the DIF-1. Although they showed that DIF-1 strongly inhibited PDE1 activity, they also reported that specific PDE1 inhibitor, 8-methoxymethyl-3-isobutyl-1-methylxanthine (IBMX), did not enhance PDE1 inhibition induced by DIF-1. Although they showed that PDE1 is a pharmacological target molecule for the DIF-1, we failed to mimic the effect of DIF-1. Moreover, we found that PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), did not induce cyclin D1 degradation (data not shown). Therefore, it is unlikely that DIFs induce cyclin D1 degradation through the inhibition of PDE1.

Recently, we reported that phosphorylation of Thr286 is a crucial event in DIF-1 action to induce cyclin D1 degradation in the squamous cell carcinoma cell line NA, since a T286A mutant of cyclin D1 was much more stable compared with a T288A mutant after DIF-1 treatment (7). In this study, we showed that both Thr286 and Thr288 residues were strongly phosphorylated by DIF-3 and T286A and T288A cyclin D1 mutants were resistant to DIF-3 treatment in HeLa cells, suggesting that not only Thr286 but also Thr288 plays an important role in DIF-3 action. This difference might be caused by the difference of the expression level of DYRK1B in NA cells and HeLa cells. To our knowledge, no studies on the expression of DYRK1B in NA cells have been reported, whereas HeLa cells have been reported to highly express DYRK1B (19).

The destruction box-like motif in cyclin D1 (Arg29-Q-X-X-Leu32) has been reported to be required for cyclin D1 degradation induced by genotoxic stress (11). To examine the involvement of this motif in DIF-3 action, the effect of DIF-3 on two different mutants (R29Q, L32A) were analyzed. We found that these mutants were fully responsive to DIF-3 treatment, indicating that the destruction box-like motif is not required for DIF-3-induced cyclin D1 degradation. This motif exists in cyclin D1 but not in cyclins D2 and D3. We reported that DIF-3 degrades not only cyclin D1 but also cyclins D2 and D3 (6). Therefore, this result is in agreement with our previous observations.

In this study, we showed that GSK-3β and DYRK1B, both of which phosphorylate cyclin D1 to induce its degradation, were involved in DIF-3 action. This may have an important implication in DIF-3-induced cyclin D1 degradation, since DIF-3 induces rapid and strong degradation of cyclin D1 (within 1 h). In tumor cells, genes that directly regulate the cell cycle are often damaged. Among them, cyclin D1 is one of the genes strongly implicated in oncogenesis (10). Amplification of the gene encoding cyclin D1 and overexpression of cyclin D1 protein have frequently been demonstrated in several types of human malignant neoplasms (26–29). Moreover, DIFs have been reported to inhibit PDE1 activity (4), and some inhibitors for PDE1 are expected to be applicable to cancer (30, 31). Therefore, it could be suggested that DIFs are potent antitumor agents and identification of the target molecule(s) for DIFs may offer ideas for the design of new anticancer drugs.

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