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Differentiation-Inducing Factor-1 Suppresses the Expression of c-Myc in the Human Cancer Cell Lines

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Abstract. Differentiation-inducing factor-1 (DIF-1), a morphogen for *Dictyostelium discoideum*, inhibits the proliferation of human cancer cell lines by suppressing the Wnt/*β*-catenin signaling pathway. In this study, we examined the effect of DIF-1 on c-Myc, a target gene product of the Wnt/*β*-catenin signaling pathway, mainly using HCT-116 colon cancer cells. DIF-1 strongly reduced the amount of c-Myc protein in time- and concentration-dependent manners and reduced c-Myc mRNA expression by inhibiting promoter activity through the TCF binding sites. The effect of DIF-1 on c-Myc was also confirmed using the human cervical cell line HeLa. Pretreatment with the proteasome inhibitor MG132 or glycogen synthase kinase-3*β* (GSK-3*β*) inhibitors (LiCl and SB216763) attenuated the effect of DIF-1, suggesting that DIF-1 induced c-Myc protein degradation through GSK-3*β* activation. Furthermore, we examined whether c-Myc was involved in the anti-proliferative effect of DIF-1 using c-Myc–overexpressing cells and found that c-Myc was associated with the anti-proliferative effect of this compound. These results suggest that DIF-1 inhibits c-Myc expression by inhibiting promoter activity and inducing protein degradation via GSK-3*β* activation, resulting in the inhibition of cell proliferation. Since c-Myc seems to be profoundly involved in accelerated proliferation of various malignant tumors, DIF-1 may have a potential to develop into a novel anti-cancer agent.

Keywords: c-Myc, Wnt/*β*-catenin signaling pathway, colon cancer cell, differentiation-inducing factor-1, glycogen synthase kinase-3*β*

Introduction

The Wnt/*β*-catenin signaling pathway is essential for proper tissue development in embryos and tissue maintenance in adults. It is well known that constitutive activation of this signaling pathway can lead to cancer development (1 – 4). There are many reports on the involvement of the Wnt/*β*-catenin signaling pathway in various cancers including colon, breast, and gastric cancers, as target genes of this signaling pathway include many proto-oncogenes such as *c-Myc* and *cyclin D1* (5 – 8).

The product of the *c-Myc* proto-oncogene is a transcriptional factor that acts in conjunction with its partner Max and has been reported to be involved in diverse cellular functions including proliferation, growth and apoptosis, as well as neovascularization (9, 10). In normal cells, c-Myc expression is tightly regulated in response to growth signals, and induction of c-Myc expression stimulates quiescent cells to enter the cell cycle (11 – 13).

Differentiation-inducing factors (DIFs) were identified in *Dictyostelium discoideum* as morphogens required for stalk cell differentiation (14, 15). In the DIF family, DIF-1 [1-(3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone] was the first to be identified. DIF activity is not limited to *Dictyostelium* and has been shown to strongly inhibit the proliferation of human cells, even in human colon cancer cell lines, in which the Wnt/*β*-catenin signaling pathway is constitutively activated (16 – 22). Previously, we reported that DIF-1 induces

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cyclin D1 protein degradation via glycogen synthase kinase-3β (GSK-3β) activation and inhibits the Wnt/β-catenin signaling pathway, thereby inhibiting cyclin D1 mRNA expression, by the suppression of transcription factor 7–like 2 (TCF7L2) expression in human colon cancer cells (22).

In this study, to further elucidate the effect of DIF-1 on downstream targets of the Wnt/β-catenin signaling pathway, we examined the effect of DIF-1 on c-Myc, a known target gene product of the Wnt/β-catenin signaling pathway and thought to play a pivotal role in cell proliferation and oncogenesis, mainly using HCT-116 human colon cancer cells.

Materials and Methods

Chemicals and antibodies

DIF-1 was synthesized as described elsewhere (14, 15). MG132 was purchased from the Peptide Institute, Osaka. SB216763 was purchased from BIOMOL International (Farmingdale, NY, USA). The anti-c-Myc monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-GAPDH monoclonal antibody was purchased from Abcam (Cambridge, UK).

Cell culture

HCT-116 human colon cancer cells (expressing wild-type APC and mutant β-catenin) and HeLa human cervical cancer cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 0.1 μg/ml streptomycin.

Western blotting

Western blotting was performed as described elsewhere (22). Samples were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane using a semidry transfer system (1 h, 12 V). Immunoreactive proteins were visualized by treatment with a detection reagent (LumiGLO; Cell Signaling Technology). Densitometric analysis was performed using NIH Image J software.

Fluorescence microscopy

Cells plated on coverslips were incubated with or without DIF-1 (30 μM) for 6 h and then washed with phosphate-buffered saline (PBS). The cells were fixed and permeabilized in ice-cold methanol/acetone (1:1) for 10 min at −20°C and then washed twice with PBS. After blocking with 2% bovine serum albumin in PBS for 1 h, the cells were incubated with an anti-GSK-3β monoclonal antibody (1:300 dilution) overnight at 4°C. The cells were washed twice with PBS and then incubated with anti-mouse IgG + IgA + IgM-biotin (Histofine, Nichirei, Tokyo) for 1 h at room temperature, followed by a streptavidin–fluorescein isothiocyanate conjugate (1:100 dilution; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Then, the cells were examined under a fluorescence microscope (Keyence, Osaka).

RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). Using 1 μg RNA, the expression of c-Myc and GAPDH mRNA was analyzed by RT-PCR. The following primers were used: c-Myc (forward: 5′-ACC CTT GCC GCA TCC ACG AAA C-3′, reverse: 5′-CCA ACA GGA ACT ATG ACC TCG ACT ACG-3′) and GAPDH (forward: 5′-ACC CTT GCC GCA TCC ACG AAA C-3′, reverse: 5′-CCA ACA GGA ACT ATG ACC TCG ACT ACG-3′).

Luciferase reporter assay

Cells were transfected with luciferase reporter plasmids and pRL-SV40, a *Renilla* luciferase expression plasmid, as a control for transfection efficiency, using Lipofectamine Plus reagent (Invitrogen). Then, cells were cultured for 24 h followed by stimulation with DIF-1 for the indicated periods. Luciferase activity was determined with a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany) and normalized to *Renilla* luciferase activity.

Construction of the c-Myc reporter plasmid

The 5′-flanking region of the human *c-Myc* gene (−1225/−1 bp relative to the transcription start site) was amplified from human genomic DNA, followed by cloning into pGL3-Basic and firefly luciferase reporter vectors. To introduce mutations in the TCF consensus binding element (TBE) (TBE1: −1187/−1181 bp, 5′-CTTT GAT-3′ and TBE2: −615/−609 bp, 5′-ATCAAAG-3′) (23), mutagenesis was carried out by PCR using c-Myc-Luc (−1225/−1 bp) as a template. The mutations (TBE1 mt: 5′-CTTTGGC-3′ and TBE2 mt: 5′-GCCAAAG-3′) were confirmed by DNA sequence analysis.

Construction of the c-Myc-overexpression plasmid

Total RNA was extracted from HCT-116 cells, and c-Myc cDNA was obtained by RT-PCR. The primer sequences for gene amplification were as follows: forward primer, 5′-TTCGGGTAGTGGAAAACCAG-3′; reverse primer, 5′-TTCCTTACGCACAAGAGTTCC-3′. The cDNA (GenBank accession number NM_002467) was verified by DNA sequencing and subcloned into pcDNA3 (Invitrogen).

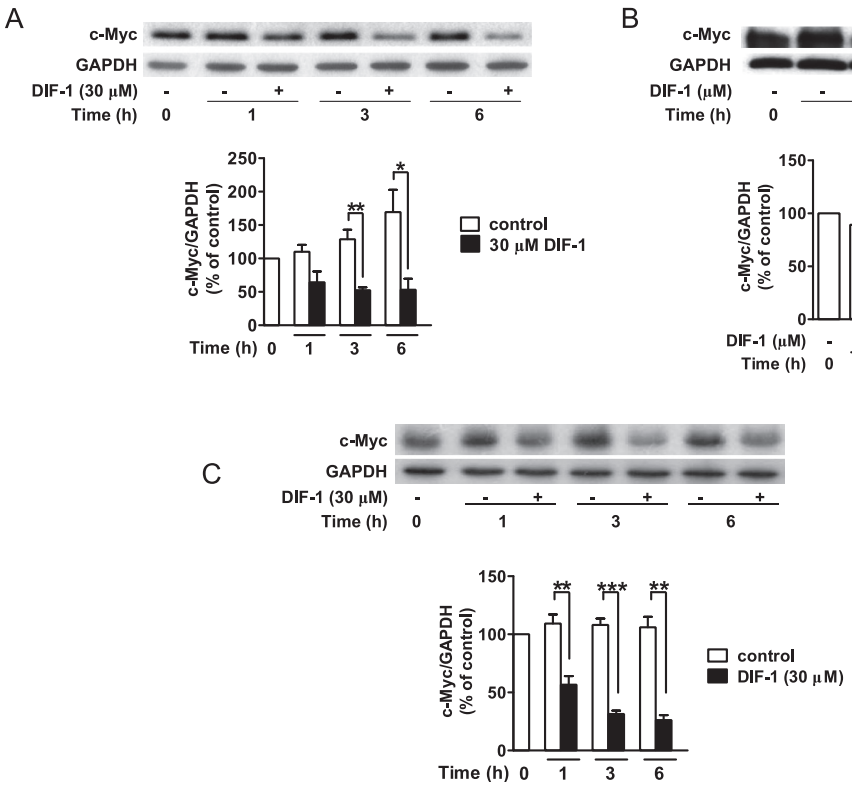

Fig. 1. DIF-1 reduces c-Myc protein level in HCT-116 and HeLa. A and C) Time course. HCT-116 cells (A) or HeLa cells (C) were incubated with or without DIF-1 (30 μM) for the indicated periods. B) Concentration-dependency. HCT-116 cells were incubated with various concentrations of DIF-1 for 6 h. Protein samples were subjected to western blot analysis using anti-c-Myc and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E.M. from three independent experiments and statistically analyzed using the Student's *t*-test (A) or one-way ANOVA with a Bonferroni post-hoc test (B). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Cell proliferation assay

HCT-116 cells (3×10^4 cells/well) were seeded in 24-well plates and treated with or without DIF-1 (10 μM) for 24 h. Cells were harvested by trypsin/EDTA treatment and enumerated.

Statistics

Results were expressed as the mean ± S.E.M. Differences between values were statistically analyzed by the Student's *t*-test or one-way ANOVA with Bonferroni post-hoc tests (GraphPad Prism 5.0; GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

DIF-1 reduces the amount of c-Myc mRNA by suppressing c-Myc promoter activity via the TBE in HCT-116 cells

Because we previously reported that DIF-1 inhibits the Wnt/β-catenin signaling pathway by reducing the amount of TCF7L2 in human colon cancer cell lines (22), and c-*Myc* is a well-known target gene of the Wnt/β-catenin signaling pathway, we analyzed the effect of DIF-1 on c-Myc protein expression using the human colon cancer cell line HCT-116. As shown in Fig. 1: A and B, DIF-1 strongly reduced the amount of c-Myc protein in time- and concentration-dependent manners. We found that DIF-1 strongly inhibited the expression of c-Myc in human cervical cancer cell line HeLa (Fig. 1C).

Next, we examined the effect of DIF-1 on c-Myc mRNA levels and found that DIF-1 reduced c-Myc mRNA expression in a time-dependent manner (Fig. 2A). Furthermore, we examined the effect of DIF-1 on c-Myc promoter activity. The 5′-flanking region of the human *c-Myc* gene was obtained by RT-PCR and cloned into a luciferase reporter plasmid (pGL3-Basic). Wild-type and mutant plasmids, which had mutations in the TBE located at −1187/−1181 bp (TBE 1: 5′-CTTTGAT-3′ → 5′-CTTTGGC-3′) and −615/−609 bp (TBE 2: 5′-ATCAAAG-3′ → 5′-GCCAAAG-3′) were used to analyze the role of the TBE in the action of DIF (Fig. 2B). As shown in Fig. 2C, wild-type c-Myc promoter activity was increased by up to approximately 1.4-fold after 24 h of incubation, whereas DIF-1 almost completely inhibited this increase. Although basal activities of the constructs, including mutant TBE 1 or TBE 2, were lower than that of the wild-type, DIF-1 showed no significant effect on the activity of these mutant constructs (Fig. 2D). These results indicated that DIF-1 decreased the c-Myc mRNA level by suppressing c-Myc promoter activity, and both TBEs (TBE1 and TBE2) were involved in the action of DIF-1.

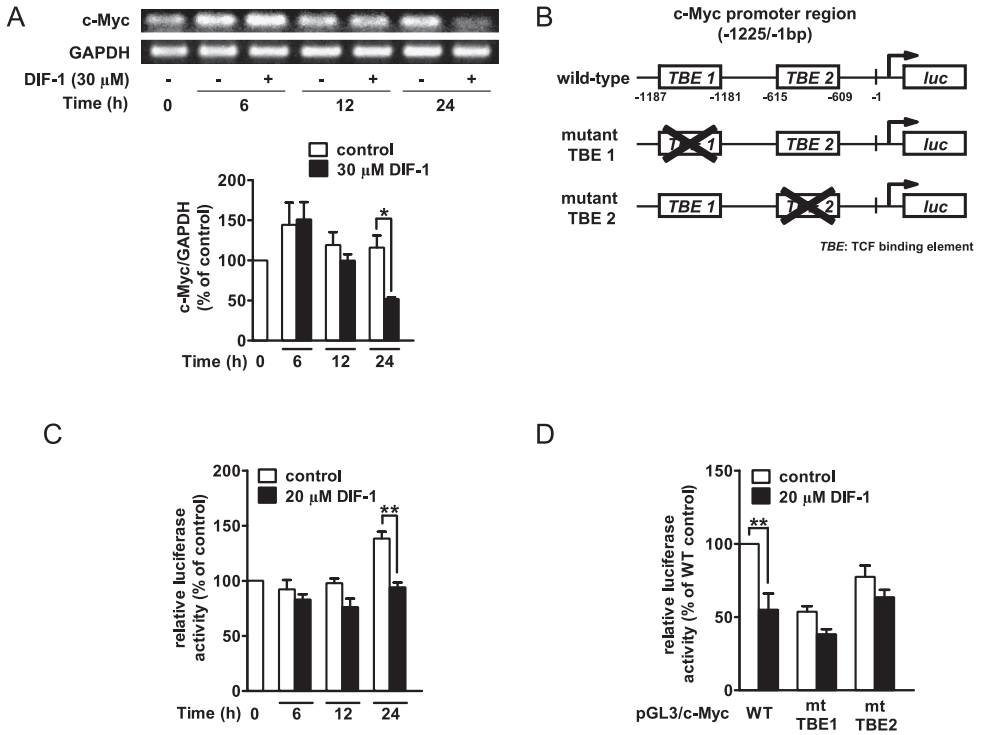

Fig. 2. DIF-1 reduces c-Myc mRNA expression and promoter activity in HCT-116 cells. A) HCT-116 cells were incubated with or without DIF-1 (30 μM) for the indicated periods. Total RNA (1 μg) was subjected to RT-PCR to analyze c-Myc and GAPDH mRNA expression. PCR cycle numbers were 21 for c-Myc and 20 for GAPDH. mRNA expression levels were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E.M. from three independent experiments and statistically analyzed using the Student's *t*-test. *$P < 0.05$. B) Schematic of pGL3-Basic–containing wild-type and TBE-mutated c-Myc promoters. C) HCT-116 cells were co-transfected with a wild-type c-Myc promoter construct (−1225/−1 bp) and pRL-SV40. After 24 h of incubation, cells were stimulated with or without DIF-1 (20 μM) for the indicated periods. Luciferase activity is shown as percentages of the control level. Values are the means ± S.E.M. from three independent experiments performed in duplicate. **$P < 0.01$. D) HCT-116 cells were co-transfected with luciferase reporter vectors (pGL3-containing wild-type or mutant c-Myc promoters) and pRL-SV40. After 24 h of incubation, cells were stimulated with or without DIF-1 (20 μM) for 24 h. Luciferase activity is shown as percentages of the wild-type control level. Values are the means ± S.E.M. from four independent experiments and statistically analyzed using one-way ANOVA with a Bonferroni post-hoc test. **$P < 0.01$.

DIF-1 induces proteolysis of c-Myc in HCT-116 cells which is mediated by GSK-3β

Because DIF-1 decreased the amount of c-Myc protein after 1 h of treatment, DIF-1 might induce not only suppression of c-Myc promoter activity but also degradation of c-Myc protein. Therefore, we used MG132, a ubiquitin-proteasome inhibitor, to analyze the action of DIF-1. As shown in Fig. 3A, pretreatment with MG132 significantly weakened the effect of DIF-1, indicating that DIF-1 rapidly reduced the amount of c-Myc protein by accelerating ubiquitin–proteasome-dependent proteolysis.

Because GSK-3β has been reported to trigger c-Myc proteolysis (24, 25), and we previously reported that DIF-1 activated this kinase, we investigated whether GSK-3β was involved in the DIF-1–induced degradation of c-Myc. Although GSK-3β is generally considered to be a cytosolic protein, GSK-3β translocates into the nucleus by various extracellular signals and subsequently phosphorylates its substrates (26, 27). Therefore, we examined the subcellular distribution of GSK-3β after DIF-1–stimulation in HCT-116 cells. Immunofluorescence staining revealed that GSK-3β was most abundant in the cytoplasm in unstimulated cells and it translocated into the nucleus after stimulation with DIF-1 (Fig. 3B), similar to that in our previous report using oral squamous cell carcinoma cell lines (20), suggesting that DIF-1 stimulated GSK-3β activity. We next examined the effect of GSK-3β inhibitors, LiCl and SB216763, on DIF-1–induced c-Myc proteolysis. As shown in Fig. 3C, pretreatment with LiCl (30 mM, 3 h) or SB216763 (20 μM, 3 h) attenuated the effect of DIF-1, indicating that GSK-3β was involved in DIF-1–induced c-Myc protein degradation in HCT-116 cells.

The effect of DIF-1 on cell proliferation is partially mediated by c-Myc protein reduction

We previously reported that DIF-1 induces cell cycle arrest at G_0/G_1 phase, leading to inhibition of cell pro-

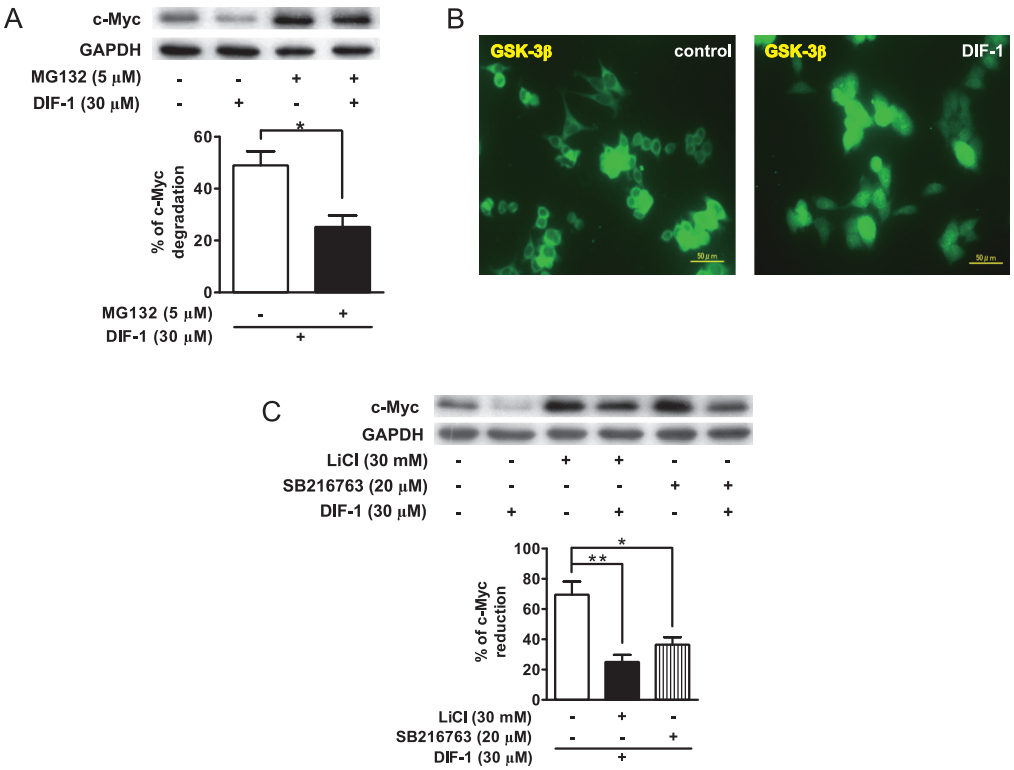

Fig. 3. GSK-3β mediates c-Myc proteolysis induced by DIF-1 in HCT-116 cells. A) Effect of the proteasome inhibitor MG132. HCT-116 cells pretreated with MG132 for 1 h were incubated with or without DIF-1 (30 μM) for 6 h. Samples were subjected to western blot analysis using anti-c-Myc and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the degraded amounts. Values are the means ± S.E.M. from three independent experiments and statistically analyzed using one-way ANOVA with a Bonferroni post-hoc test. *$P < 0.05$. B) Immunofluorescence. Cells were plated on coverslips and incubated with or without DIF-1 (30 μM) for 6 h. Immunofluorescence staining was performed with an anti-GSK-3β monoclonal antibody. The results are representative of four independent experiments. C) HCT-116 cells were pretreated with or without LiCl (30 mM) or SB216763 (20 μM) for 3 h and then incubated with or without DIF-1 (30 μM) for 6 h. Protein bands were quantified and shown as percentages of the reduced amounts. Values are the means ± S.E.M. from four independent experiments and statistically analyzed using one-way ANOVA with a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$.

liferation in several cancer cell lines including HCT-116 (17, 18, 20 – 22). It has been reported that c-Myc is a positive regulator of the G_1 phase by activating G_1 phase–specific cyclin-dependent kinases (Cdks) and cyclins (28 – 30). To elucidate whether c-Myc was involved in the anti-proliferative effect of DIF-1, we examined the effect of DIF-1 using c-Myc–overexpressing HCT-116 cells. As shown in Fig. 4A, although the expression level of c-Myc in cells transfected with empty pcDNA3 was reduced after 24 h of stimulation with DIF-1, it was not significantly changed in cells transfected with pcDNA3/c-Myc. Next, we examined the effect of DIF-1 on cell proliferation using cells transfected with empty pcDNA3 or the pcDNA3/c-Myc vector. Compared with cells transfected with empty pcDNA3, the effect of DIF-1 on cell proliferation was partially attenuated in c-Myc–overexpressing cells (Fig. 4B). These results indicated that the reduction of c-Myc was associated with the anti-proliferative effect of DIF-1 in HCT-116 cells. We also tried to examine the effect of MG132 or GSK-3β inhibitors (LiCl and SB216763) on the anti-proliferative effect of DIF-1 to clarify the involvement of c-Myc reduction. However, all of these agents strongly inhibited cell proliferation (Fig. 4C), so we could not assess the effect of these agents on the effect of DIF-1.

Discussion

We previously reported that DIF-1 shows an anti-tumor effect by suppressing the expression of cyclin D1 and the Wnt/β-catenin signaling pathway. In this study, to further elucidate the effect of DIF-1 on downstream targets of the Wnt/β-catenin signaling pathway, we examined c-Myc using HCT-116 human colon cancer cells. We found that DIF-1 suppressed c-Myc mRNA expression by inhibiting promoter activity through the TBEs and induced c-Myc protein degradation via GSK-3β activation.

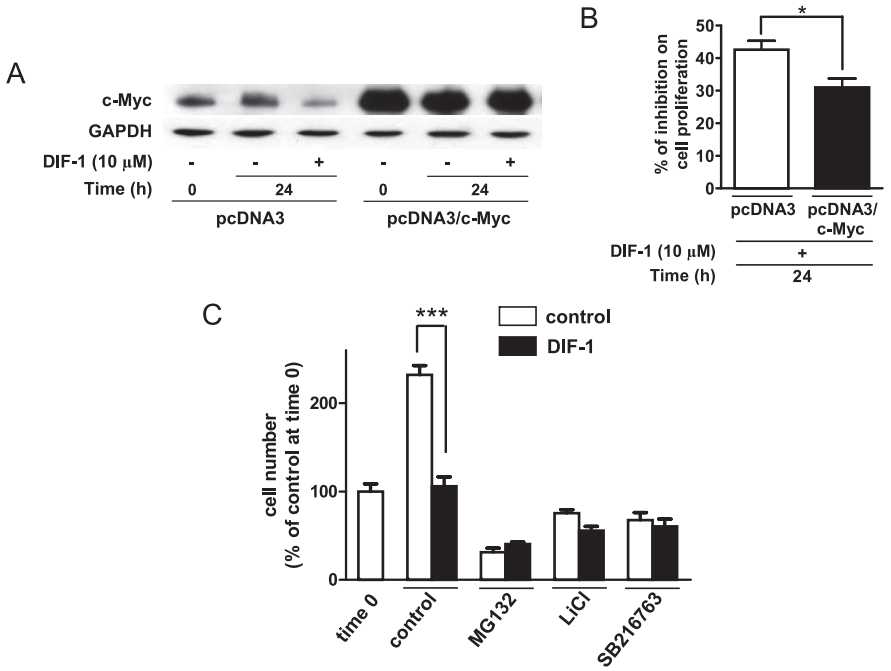

Fig. 4. The effect of DIF-1 on cell proliferation is partially weakened by c-Myc overexpression. A) pcDNA3 or pcDNA3/c-Myc were transfected into HCT-116 cells. After 24 h of incubation, cells were stimulated with or without DIF-1 (10 μM) for 24 h. Protein samples were subjected to western blot analysis using anti-c-Myc and anti-GAPDH antibodies. The results are representative of four independent experiments. B) Cell proliferation assay. pcDNA3 or pcDNA3/c-Myc were transfected into HCT-116 cells. After 24 h of incubation, cells were stimulated with or without DIF-1 (10 μM) for 24 h. Cells were harvested by trypsin/EDTA treatment and counted. Data are shown as percentages of the reduced numbers. Values are the means ± S.E.M. from five independent experiments. *$P < 0.05$ vs. control. C) HCT-116 cells were seeded on a 24-well plate. After 24-h incubation (this time point was set as time 0), cells were pretreated with or without MG132 (5 μM), LiCl (30 mM), or SB216763 (20 μM) for 3 h and then incubated with or without DIF-1 (30 μM) for 24 h. Cells were harvested by trypsin/EDTA treatment at the indicated periods and then counted. Data are shown as percentages of the control at time 0. Values are means ± S.E.M. from three independent experiments done in triplicate. ***$P < 0.001$ vs. control.

In the previous report, we showed that DIF-1 reduced cyclin D1 promoter activity through TBEs by inhibiting TCF7L2 expression in HCT-116 cells without affecting β-catenin protein expression (22). Although we did not examine the effect of DIF-1 on the amount of β-catenin or TCF7L2 protein in the present study, the DIF-1–induced suppression of c-Myc promoter activity through TBEs may have been caused by the same mechanism as DIF-1 suppression of cyclin D1 promoter.

As shown in Fig. 3B, DIF-1 activated GSK-3β and induced translocation of this kinase into nuclei, which was consistent with our previous report (20). Activity of GSK-3β is regulated by the phosphorylation status of Ser^9 and Akt is one of the known protein kinases that phosphorylate the Ser^9 of GSK-3β. Akt inhibits the activity of GSK-3β by phosphorylating the Ser^9. It is also known that Akt is constitutively activated, due to mutations in the upstream kinase, phosphatidylinositol 3-kinase (PI3K), in HCT-116 cells (31). Therefore, the suppression of Akt activity may not be the mechanism for the DIF-1–induced activation of GSK-3β, although we have not succeeded in identification of that mechanism.

We also revealed that c-Myc overexpression partially attenuated the anti-proliferative effect of DIF-1. This result clearly indicated that not only reduction of cyclin D1 but also that of c-Myc played an important role in the anti-tumor effect of DIF-1. Since induction of c-Myc expression stimulates quiescent cells to enter the cell cycle by activating the expression of cell division cycle 25A (CDC25A) and subsequent activation of cyclin D1–Cdk 4/6 and the cyclin E–Cdk2 complex to promote G_1 to S phase progression (11 – 13), reduction of c-Myc may cause cell cycle arrest. We previously reported that DIF-1 arrested the cell cycle at G_0/G_1 phase in HCT-116 cells and thought it was caused by the reduction of cyclin D1 expression (22). However, the present result suggests a part of this effect might be caused by the reduction of c-Myc. In spite of the overwhelming levels of c-Myc expression, the reversal of anti-proliferative effect of DIF-1 was only partial (approx. 10%). This is probably because cyclin D1 was still reduced in those cells by DIF-1 treatment.

Because of gene amplification, chromosomal translocation and constitutive activation of upstream signaling

pathways such as Wnt/β-catenin, MAPK, and Notch, *c-Myc* gene expression is observed in virtually every human cancer (11 – 13). Therefore, the development of the agents that inhibit c-Myc activity/expression may bring about great advance in medical cancer therapeutics. In fact, a number of anti-c-Myc strategies are currently being explored for the treatment of cancer (10). Because DIF-1 strongly suppressed the expression of c-Myc protein, this compound could have a potential to develop into a novel anti-cancer agent.

Acknowledgments

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