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Overexpression of DA41 in v-Ha-*ras*-3Y1 Cells Causes Growth Suppression

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We have recently found that DA41 exhibits marked homology with mouse PLIC-1, PLIC-2, frog XDRP1 and yeast DSK2. XDRP1 has been shown to be associated with cyclin A, and blocks cell division of frog embryo. In the present study, we examined the biological role(s) of DA41 in mammalian cells by overexpressing it in v-Ha-*ras*-transformed 3Y1 cells (*ras*-3Y1). Transfectants which expressed a high level of *DA41* mRNA exhibited a decrease in growth rate, a reduction in saturation density, and a suppression of colony formation in soft agar medium. To clarify the molecular mechanism(s) by which DA41 affects cell growth, the effect of DA41 expression on the levels of various cell cycle-regulatory proteins was examined. The forced expression of *DA41* gene resulted in a remarkable reduction in CDK2 activity, while the amount of CDK2 did not change. These observations indicate that DA41 is involved in cell cycle regulation in *ras*-3Y1 cells.

Key words: DA41 — Growth suppression — *ras* — 3Y1

We have previously shown that rat DAN protein suppresses the malignant phenotypes of v-*src*-transformed 3Y1 cells (SR-3Y1) and also negatively regulates cell cycle progression at the G1/S boundary.[1, 2] DAN is a secreted glycoprotein[3, 4] with a characteristic cysteine-knot structure common to the DAN/Cerberus family, which includes Gremlin/Drm.[5] Injection studies in frog embryos have suggested that the DAN/Cerberus family acts as an antagonist regulating BMP signalling.[5]

DA41 was initially identified as a novel cytoplasmic protein which can associate with DAN by means of yeast two-hybrid screening of a normal rat lung cDNA library.[6] The interaction between DAN and DA41 is mediated through the NH_2-terminal domain and the cysteine-knot region of DAN. The expression of *DA41* gene was ubiquitous in adult rat tissues. Interestingly, the amount of *DA41* mRNA started to increase at the late G1 phase and the same level was maintained until the onset of the S phase of the cell cycle, raising the possibility that DA41 might have some regulatory role(s) in cell cycle progression.[6] Additionally, we have reported that human *DA41* was mapped to chromosome 9q21.2-q21.3, a position overlapping the candidate tumor suppressor locus for bladder cancer.[7]

A recent search of the databases for DA41-related protein(s) identified mouse PLIC-1, PLIC-2, frog XDRP1 and yeast DSK2.[7–10] PLIC-1 and PLIC-2 interact with the cytoplasmic tail of integrin-associated protein (IAP) and mediate the interaction between IAP and vimentin-containing intermediate filaments.[8] On the other hand, XDRP1 associates with the NH_2-terminal region of cyclin A and inhibits the degradation of cyclin A.[9] Microinjection of recombinant XDRP1 into fertilized frog eggs blocked the cell division, suggesting a cell cycle-regulatory role of XDRP1.[9] Furthermore, overproduction of DSK2 induced a mitotic defect with a short spindle.[10]

In the present study, we examined the role(s) of DA41 in mammalian cells by overexpressing it in v-Ha-*ras*-transformed 3Y1 cells (*ras*-3Y1). As described previously, the expression of *DA41* was significantly lower in *ras*-3Y1 cells compared with that in the parental rat fibroblast 3Y1 cells.[6] Overexpression of DA41 caused a significant suppression of transformed phenotypes of *ras*-3Y1 cells, accompanied with a remarkable reduction of CDK2 activity. These results indicate that DA41 might participate in the growth-regulatory machinery.

MATERIALS AND METHODS

Cell culture and transfection Rat fibroblast 3Y1 and v-Ha-*ras*-transformed 3Y1 (*ras*-3Y1) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

A rat *DA41* cDNA, which contains the entire open reading frame (ORF), was inserted into the *Eco*RI site of the mammalian expression vector pMEXneo in a forward orientation (pMEXneo-*DA41*). For transfection, *ras*-3Y1 cells were subcultured to 50% confluency 1 day before transfection. Ten micrograms of pMEXneo-*DA41* and 30 μg of lipofectin reagent were used following the protocol from GIBCO-BRL (Grand Island, NY). Cells were incubated for 12 h in serum-free medium containing DNA and lipofectin, and then transferred to growth medium containing G418 at a concentration of 400 μg/ml. After 2 weeks

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of culture, G418-resistant colonies were picked up with a cloning cylinder and expanded into cell lines.

β-Galactosidase assay *ras*-3Y1 cells (at a density of 5×10^5 cells/dish) were transfected with either an empty vector (pMEXneo) or pMEXneo-*DA41* together with the reporter plasmid (pCH110), which encoded β-galactosidase, by using the standard calcium phosphate-DNA precipitation procedure.[11] Twelve or 24 h after transfection, cells were washed twice with 1× PBS (10 m*M* sodium phosphate, pH 7.2, 150 m*M* NaCl) and fixed with 0.25% glutaraldehyde for 10 min at 4°C. Cells were then washed twice with 1× PBS and soaked in the solution containing 5 m*M* $K_4[Fe(CN)_6]3H_2O$, 5 m*M* $K_3Fe(CN)_6$, 200 μM $MgCl_2$, 10 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for 12 h at 37°C. Cells were washed four times with 1× PBS and fixed with 3.7% formaldehyde for 30 min at room temperature, and the numbers of β-galactosidase-positive cells were scored.

Northern blot hybridization Ten micrograms of total cellular RNA prepared by the guanidine thiocyanate method[12] was separated on 1% agarose gel containing formaldehyde, transferred to nylon membrane in 20× SSC, and immobilized by UV-cross-linking. A hybridization probe was prepared from a gel-purified DNA fragment which was radio-labeled by random priming with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP. The blot was hybridized in a solution containing 6× SSC, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 50% deionized formamide, and 100 μg/ml of heat-denatured salmon sperm DNA at 42°C for 24 h. After the hybridization, the blot was washed twice in 2× SSC, 0.1% SDS at room temperature and twice in 0.1× SSC, 0.1% SDS at 50°C.

Soft agar colony growth assay The cells (2.5×10^3) were suspended in 3 ml of 0.375% low-melting-point agarose (SeaPlaque; FMC, Rockland, MA) dissolved in culture medium and plated onto an agarose bed consisting of 0.53% low-melting-point agarose and the same medium.[1] After 2 weeks, visible colonies larger than 100 μm were scored.

Cell cycle analysis Cells were seeded at 5.0×10^5 cells/10-cm dish. The medium was replaced 24 h later with fresh medium. After an additional 24 h, cells were harvested, fixed in 80% ethanol and treated with propidium iodide.[13] Flow cytometric analysis was performed with a Becton Dickinson FACScan flow cytometer. In order to evaluate the significance of the differences, the χ^2 test was carried out. *P* values less than 0.05 were considered statistically significant.

Western analysis Cells were washed with 1× PBS, collected by scrapping and directly dissolved in protein lysis buffer containing 0.5% NP-40, 20 m*M* Tris-Cl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA [*O*,*O*′-bis(2-aminomethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid] and 1 m*M* PMSF (phenylmethylsulfonyl fluoride) (for Ras, c-Raf1 and ERK2) or containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 0.1 m*M* PMSF (for CDK2 and $p27^{kip1}$). Lysates were centrifuged for 20 min at 4°C to remove insoluble cellular debris, mixed with SDS sample buffer (62.5 m*M* Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.001% BPB), and separated by electrophoresis in a 10% SDS-polyacrylamide gel.[14] After electrophoresis, proteins were electro-transferred to a nitrocellulose membrane for 1 h. The membrane was blocked in TBS (100 m*M* Tris-Cl, pH 7.5, 150 m*M* NaCl) containing 5% nonfat dried milk and 0.05% Tween 20 for 12 h. The membrane was incubated with anti-Ras (Seikagaku Corp., Tokyo), anti-c-Raf1 (Transduction Laboratories, Lexington, KY), anti-ERK2 (Transduction Laboratories), anti-CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-$p27^{kip1}$ (Santa Cruz Biotechnology) in TBS containing 0.05% Tween 20 for 1 h at room temperature and then washed with TBST solution (TBS containing 0.05% Tween 20) for 30 min. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (GIBCO-BRL) was then applied to the membrane for 1 h in TBST. The protein of interest was then visualized by using chemiluminescence (ECL) reagent (Amersham, Arlington Heights, IL) followed by exposure to an X-ray film.

***In vitro* kinase reaction** The cell lysate containing 400 μg of protein was treated with 50 μl of protein A/protein G Sepharose on a rotator for 30 min at 4°C and the Sepharose beads were pelleted by centrifugation for 5 min. The supernatant was then mixed and incubated with 1 μg of anti-CDK2 (Santa Cruz Biotechnology) for 2 h at 4°C. Then 20 μl of protein A/protein G Sepharose was added to the reaction mixture and the whole was incubated overnight at 4°C. The Sepharose beads were recovered by centrifugation, washed twice with lysis buffer, three times with kinase buffer [50 m*M* Tris-Cl, pH 7.2, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT)], and resuspended in 25 μl of kinase buffer containing 50 μg/ml histone H1 (Boehringer Mannheim, GmbH, Germany). The kinase reaction was allowed to proceed for 30 min at 30°C in the presence of [γ-^{32}P]ATP and was stopped by the addition of SDS sample buffer. After having been boiled for 5 min, the mixture was analyzed on a 10–20% SDS-polyacrylamide gradient gel. The gel was then dried and exposed to an X-ray film.[15]

RESULTS

Ectopic expression of *DA41* gene As described previously, the expression of *DA41* gene was sharply down-regulated in v-Ha-*ras*-transformed 3Y1 cells (*ras*-3Y1).[6] In order to examine the biological role(s) of DA41 in mammalian cells, we constructed an expression vector encod-

ing rat DA41 (pMEXneo-*DA41*) and *ras*-3Y1 cells were transiently transfected with empty vector (pMEXneo) or pMEXneo-*DA41* together with a reporter plasmid encoding β-galactosidase (pCH110) to identify transfected cells. Numbers of β-galactosidase-positive cells were scored at 12–48 h post transfection. As shown in Fig. 1, overexpression of DA41 resulted in a remarkable growth inhibition of the recipient cells. In order to analyze molecular event(s) induced by the overexpression of DA41, we generated stable transfectants (R3, R5 and R6) which constitutively expressed *DA41* mRNA in *ras*-3Y1 cells (Fig. 2). As described previously, two specific *DA41* transcripts (2.4 and 3.2 kb) were detected in 3Y1 and also in various transformed 3Y1 cells.[6] Since the *DA41* cDNA (approximately 2.2 kb in length) used in this study might be derived from the 2.4 kb transcript, the level of the 2.4 kb mRNA was expected to be increased by the transfection. The largest amount of *DA41* mRNA was detected in R6 cells. As the levels of *DA41* mRNA in two other clones (R1 and R2) were the same as that of *ras*-3Y1 cells, we used them as negative controls in the experiments described below.

Reversion of the transformed phenotypes by DA41-overexpression As shown in Fig. 3, a significant morphological reversion was observed in R6 cells, while the morphological phenotype was retained in the control transfectants (R1 and R2). No clear morphological changes could be observed in R3 and R5 cells. We then examined whether the increased level of DA41 is directly related to the inhibition of cell growth. As shown in Table I, both the growth rate and the saturation density of the DA41-overexpressing cells (R3, R5 and R6) were reduced compared with those of the parental *ras*-3Y1 cells and the control transfectants. Most importantly, the ability to grow in soft agar was significantly decreased in these DA41-overexpressing cells.

Cell cycle distribution of DA41-overexpressing cells Asynchronous cultures of the transfectants, 3Y1 and *ras*-3Y1 cells were collected and their cell cycle distributions were analyzed by flow cytometry. The G1 fraction observed in 3Y1 cells was 46.5%, whereas the rapidly growing cells (*ras*-3Y1, R1 and R2) exhibited decreased G1 fractions (Table II). Interestingly, the stable transfectants that overexpressed DA41 displayed a significant increase in the percentage of cells at the G1 phase (47–51% G1).

Effects of DA41 overexpression on the levels of growth regulators As the ectopic overexpression of DA41 suppressed the growth of *ras*-3Y1 cells, it is important to investigate whether DA41 overexpression modulates the levels of Ras and molecules involved in its downstream signalling. Western blot analysis revealed that the levels of Ras, c-Raf1 and ERK2 (extracellular signal-regulated kinase 2) were little changed by the constitutive expression of the *DA41* gene (Fig. 4). The results obtained in the cell cycle analysis indicated that the initiation of S phase was retarded in the presence of a large amount of DA41.

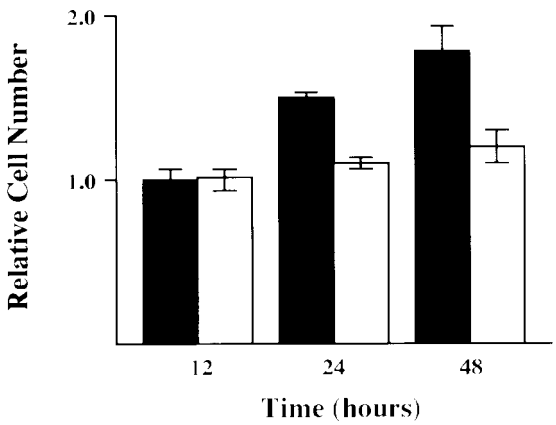

Fig. 1. Overexpression of DA41 suppresses growth of *ras*-3Y1 cells. *ras*-3Y1 cells were transfected either with an empty vector (pMEXneo) or with pMEXneo-*DA41* together with the reporter plasmid pCH110, which contains *β-galactosidase* gene. At the indicated times after the transfection, transfected cells were fixed with 0.25% glutaraldehyde and detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The numbers of stained cells were scored. Solid and open boxes indicate the relative number of β-galactosidase-positive cells transfected with pMEXneo and pMEXneo-*DA41*, respectively.

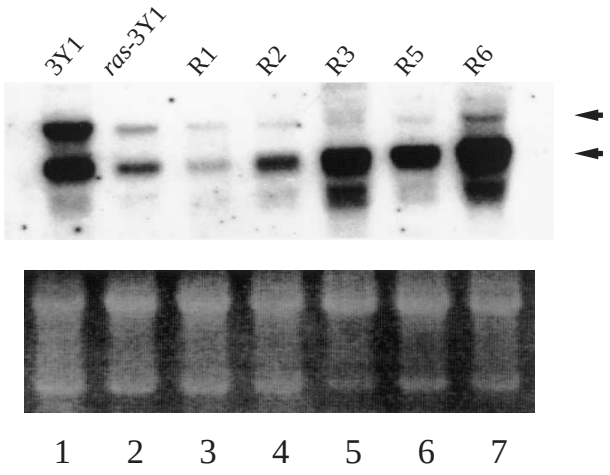

Fig. 2. Overexpression of *DA41* gene in *ras*-3Y1 cells. Total RNA was extracted from each cell line, electrophoresed on a 1% formaldehyde agarose gel, and hybridized with a random-primed ^{32}P-labeled rat *DA41* cDNA. Equal loading of RNA was confirmed by ethidium bromide staining. Arrowheads indicate *DA41* mRNA. 3Y1 (lane 1), *ras*-3Y1 (lane 2), R1 (lane 3), R2 (lane 4), R3 (lane 5), R5 (lane 6) and R6 (lane 7).

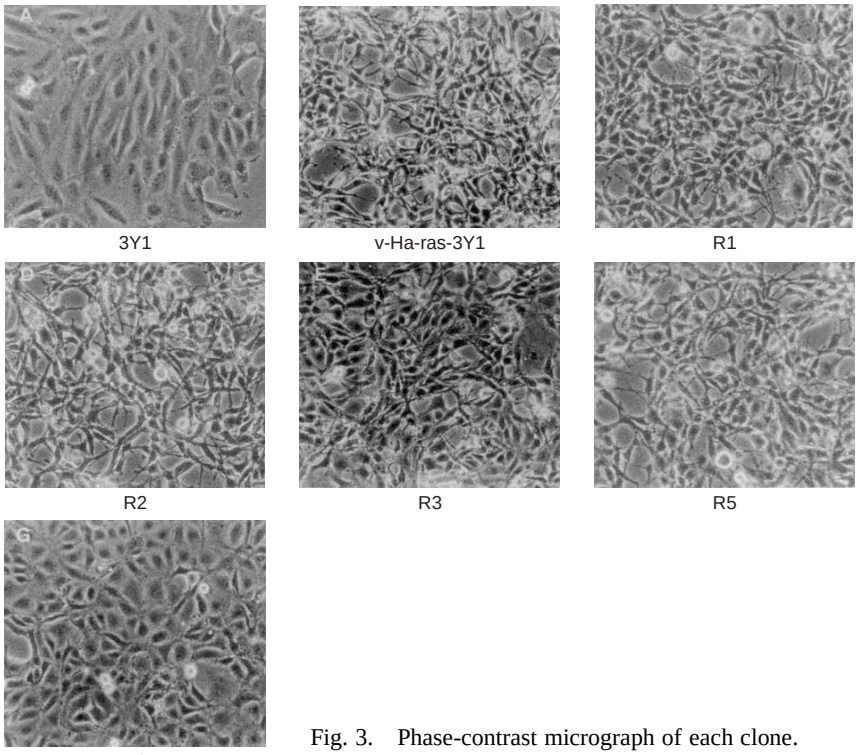

Fig. 3. Phase-contrast micrograph of each clone.

Table I. Growth Properties of Each Clone

Cell line	Doubling time (h)[a)]	Saturation density[a)] (10^6 cells)	Growth efficiency[b)] in soft agar (%)
3Y1	18.0	1.6	0
ras-3Y1	14.8	14.0	44.4
R1	16.5	11.0	43.4
R2	15.1	12.0	37.0
R3	20.6	8.0	7.7
R5	20.5	7.5	10.2
R6	20.6	6.5	3.4

a) Doubling time was determined by calculating the growth rate of exponentially growing cells. Saturation density is the number of cells per 35-mm dishes after the culture had reached confluency. Each experiment was carried out at least twice.
b) A total of 2.5×10^3 cells was suspended in 0.375% low-melting-point agarose (SeaPlaque; FMC) dissolved in culture medium and layered onto an agarose bed consisting of 0.53% low-melting-point agarose and the same medium. Colonies (larger than 100 μm) were counted after 14 days of growth. Growth efficiency was expressed as a percentage of the number of colonies formed in soft agar. Each value is the mean of three dishes for each cell line.

Therefore, we tested whether the protein levels of CDK2 (cyclin-dependent protein kinase 2) and $p27^{kip1}$ and the activity of CDK2 change in DA41-overexpressing clones.

Table II. Effects of *DA41* Overexpression on Asynchronous Cells[a)]

Cells	G1[b)]	S	G2/M
3Y1	46.5	41.0	12.5
ras-3Y1	35.5	48.0	16.5
R1	35.5	47.0	17.0
R2	36.5	48.0	15.0
R3	48.0	39.5	12.0
R5	47.0	38.0	14.5
R6	51.0	33.5	15.0

a) Cells were seeded at 5.0×10^5 cells/10-cm dish. The medium was replaced 24 h later with fresh medium. After an additional 24 h, cells were trypsinized and cytometric analysis was performed.
b) *P* values were calculated according to the χ^2 test. Those for 3Y1 versus *ras*-3Y1, R1 and R2 were 0.0012, 0.0012 and 0.0031, respectively. Those for *ras*-3Y1 versus R3, R5 and R6 were 0.0003, 0.0007 and <0.0001, respectively.

As shown in Figs. 5 and 6, the levels of CDK2 and $p27^{kip1}$ were unchanged among the transfectants, though a remarkable reduction in CDK2 activity was detected in DA41-overexpressing transfectants compared with the parental cells and the control transfectants.

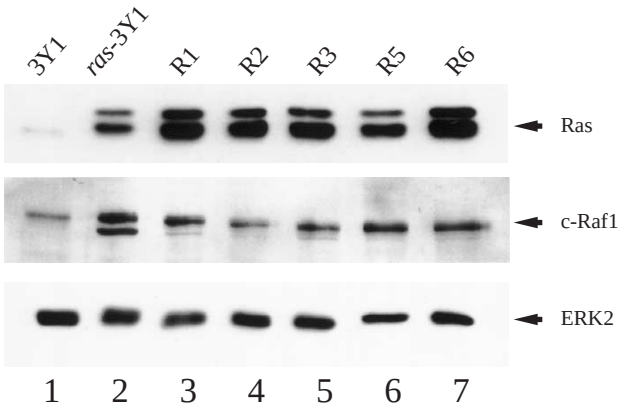

Fig. 4. Effect of *DA41*-overexpression on growth-regulatory proteins. Equal amounts of cell lysates (containing 20 μg protein) were subjected to western blot analysis as described in "Materials and Methods." The positions of Ras, c-Raf1 or ERK2 are indicated by arrowheads. The weak signal detected by anti-Ras antibody in 3Y1 cells corresponds to c-Ras. 3Y1 (lane 1), *ras*-3Y1 (lane 2), R1 (lane 3), R2 (lane 4), R3 (lane 5), R5 (lane 6) and R6 (lane 7).

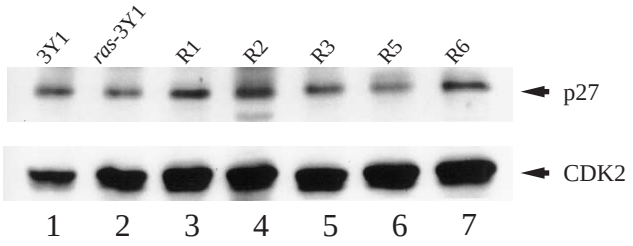

Fig. 5. Western blot analysis of CDK2 and $p27^{kip1}$. For western analysis, total cell extracts were separated by SDS-PAGE and immunoblotted with an anti-CDK2 or an anti-$p27^{kip1}$ antibody. The position of CDK2 or $p27^{kip1}$ is indicated by an arrowhead. 3Y1 (lane 1), *ras*-3Y1 (lane 2), R1 (lane 3), R2 (lane 4), R3 (lane 5), R5 (lane 6) and R6 (lane 7).

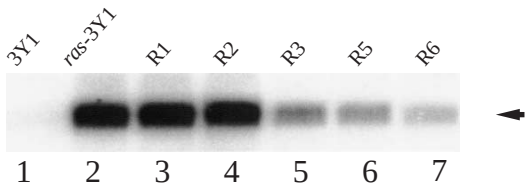

Fig. 6. CDK2 activity against histone H1. For *in vitro* kinase reaction, cells were collected and the total cell extracts (containing 400 μg protein) were subjected to immunoprecipitation with an anti-CDK2 antibody followed by histone H1 assays as described in "Materials and Methods." The phosphorylated histone H1 is indicated by an arrowhead. 3Y1 (lane 1), *ras*-3Y1 (lane 2), R1 (lane 3), R2 (lane 4), R3 (lane 5), R5 (lane 6) and R6 (lane 7).

DISCUSSION

When *DA41* gene was overexpressed in *ras*-3Y1 cells, a remarkable growth suppression was observed in the recipient cells. Colony formation efficiency in soft agar medium was significantly reduced in the stable transfectants, which expressed a large amount of *DA41* mRNA. Cell cycle analysis demonstrated that *DA41*-overexpression was accompanied with an increase in the percentage of cells at the G1 phase of the cell cycle, suggesting that DA41 can inhibit the cell cycle progression from G1 to S phase. It is intriguing to note that only R6, which exhibited the highest level of *DA41* expression, showed morphological reversion suggesting that the threshold level of DA41 to counteract the transformed phenotype is higher than that of other cell growth-related factors.

The mammalian cell cycle is regulated by the sequential activation of cyclin-dependent kinases (CDKs).[16] The activity of CDKs is dependent on cyclin binding and phosphorylation mediated by the CDK-activating kinase (CAK).[17–19] Alternatively, CDK activity is regulated by interaction with CDK inhibitors.[20] The active cyclin E-CDK2 and cyclin A-CDK2 complexes appear during the G1 phase and the G1/S transition, respectively.[21] The inhibition of CDK2 complexes may be sufficient for cell cycle arrest in a variety of cells.[22] Blain *et al*. reported that $p27^{kip1}$ can interact with cyclin A-CDK2 complex and inhibit its kinase activity, whereas the activity of CDK4 or CDK6 complex is not inhibited by $p27^{kip1}$.[22] In this study, we found that DA41-induced growth suppression in *ras*-3Y1 cells was correlated with a remarkable reduction of CDK2 activity. This down-regulation of CDK2 activity was not correlated with a change in the amount of CDK2 or $p27^{kip1}$. As described previously, CDK2 is phosphorylated by CAK, leading to an increase in its histone H1 kinase activity.[23] In our experiments, CDK2 was detected as a doublet band, which may be generated by the phosphorylation by CAK. This occurred irrespective of the overexpression of DA41 (Fig. 5), indicating that DA41-mediated down-regulation of CDK2 activity could not be explained in terms of the phosphorylation state of CDK2. Additionally, CDK2 in control transfectants might be phosphorylated by CAK to generate histone H1 kinase activity, suggesting that DA41 inhibits CDK2 after the onset of CDK2 activation. Recently, Chen and Hitomi reported that VP16-induced growth arrest of v-*src*-transformed fibroblasts was associated with a decrease in CDK2 kinase activity.[24] In their experimental systems, the overall levels of CDK2 and $p27^{kip1}$ did not change upon treatment with VP16, though there was a significant

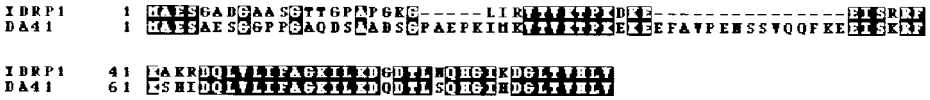

Fig. 7. Structural similarity of DA41 to XDRP1 within the N-terminal ubiquitin-like domains. Identical amino acids are printed in white type on a black background. Gaps were introduced to maximize the alignment.

decrease in the amount of cyclin A-associated CDK2. Although the exact molecular mechanism(s) involved in the down-regulation of CDK2 activity in *DA41*-transfected cells is unclear, dissociation of cyclin A from CDK2 complex might contribute to the significant reduction of CDK2 activity in *DA41*-transfected cells. Interestingly, Funakoshi *et al*. identified a frog protein, XDRP1, that can bind to cyclin A and inhibit the degradation of cyclin A.[9] XDRP1 showed marked sequence homology with DA41[7] and overexpression of XDRP1 in frog embryos blocked embryonic cell division.[9] The N-terminal ubiquitin-like domain of XDRP1 (amino acid residues 1–76) was necessary for the interaction with cyclin A. The corresponding region of DA41 was highly related to that of XDRP1 (Fig. 7), indicating that DA41 possesses a function similar to that of XDRP1. The rapid degradation of cyclins, which is mediated by the ubiquitin-dependent proteasome pathway, is required for cell cycle progression.[25] It is possible that, like XDRP1, DA41 can block the degradation of cyclin A and inhibit the cell cycle progression. In this connection, it would be important to examine whether the interaction of DA41 with cyclin A results in the dissociation of cyclin A from CDK2.

As described previously, DA41 was initially discovered as a DAN-binding protein using a yeast two-hybrid screening strategy.[6] Recent work in our laboratory demonstrated that the majority of DAN is secreted into the culture medium and might act extracellularly to exert its growth-suppressive activity.[3] Interestingly, Hsu *et al*. found that DAN can associate with BMP2 and antagonize its activity in frog early embryos.[5] However, we have not been able to detect a direct interaction between DAN and DA41 in mammalian cells. Considering that DAN acts outside the cells, the DA41-induced growth inhibition observed in *ras*-3Y1 cells might take place in the absence of physical interaction with DAN. Studies are under way to elucidate the precise function of DA41 and the molecular mechanism of the DA41-mediated inhibition of CDK2 activity.

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