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Transcriptional Regulation of the *Drosophila* Proliferating Cell Nuclear Antigen Gene and *raf* Proto-oncogene by Ursolic Acid in *Drosophila* Cultured Kc Cells

Thae Yeong Choi, Sang Hoon Rhee, Han Do Kim, Chong-Rak Kim[1], Ho Sung Kang, and Mi Ae Yoo*

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea;
[1]Department of Biology, College of Natural Sciences, Inje University, Kimhae 621-749, Korea

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Promoter of the *Drosophila* proliferating cell nuclear antigen (PCNA) gene contains DRE (*Drosophila* DNA replication-related element) required for the high level expression of replication-related genes. Recently, we found that promoter region of the *D-raf* (a *Drosophila* homolog of the human *c-raf-1*) contains two sequences homologous to the DRE and demonstrated the DRE/DREF (DRE-binding factor) involvement in regulation of the *D-raf* gene. In this study, using ursolic acid (UA), a pentacyclic triterpene acid reported to possess antitumor activities, we examined effects of UA on proliferation of the *Drosophila* cultured Kc cells and on expression of the PCNA and *D-raf* genes. UA showed an inhibitory effect on proliferation of the Kc cells in a concentration-dependent manner in DNA content assays and [^{3}H]thymidine incorporation assays. The IC_{50} value of anti-proliferative effects of UA in DNA content assays was about 7.5 μM. UA showed inhibitory effects on expression of the PCNA as well as on that of the *D-raf*, which were examined with the reporter plasmid p5'-168DPCNACAT or p5'-878DrafCAT, respectively. The results obtained in the present study suggest that expression of the PCNA and *D-raf* genes is coordinately regulated in at least UA-treated Kc cells and that down-regulation of expression of the PCNA and *D-raf* genes might be related with the anti-tumor activities of UA.

Proliferating cell nuclear antigen (PCNA) was first described as a nuclear antigen, restricted to proliferating cells (Miyachi et al., 1978). PCNA has been identified as a cofactor of DNA polymerase δ (Bravo et al., 1987; Prelich et al., 1987a) and is required for cellular DNA synthesis (Bravo et al., 1987; Prelich et al., 1987b) and for cell cycle progression (Jaskulski et al., 1988; Liu et al., 1989). cDNAs for *Drosophila* PCNA have been cloned and completely sequenced (Yamaguchi et al., 1990).

Raf-1 is a protein serine/threonine kinase located primarily in the cytosol (Morrison, 1990; Rapp, 1991). The Raf-1 serves as a central intermediate in many signaling pathways, ultimately regulating cell proliferation, differentiation, and development (Crews and Erickson, 1993; Davis, 1993) by connecting upstream tyrosine kinase with downstream serine/threonine kinases such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) (Roberts, 1992; Crews and Erickson, 1993). A *Drosophila* homolog of the human *c-raf-1*, the *D-raf* has been cloned and mutants for this gene have been identified (Nishida et al., 1988; Ambrosio et al., 1989; Hata et al., 1994). *D-raf* is also required for the regulation of cell proliferation and differentiation (Perrimon et al., 1985; Nishida et al., 1988; Melnick et al., 1993; Hata et al., 1994). However, little is known about the regulation of *raf* proto-oncogene expression.

Promoter of the *Drosophila* PCNA gene contains DRE (*Drosophila* DNA replication-related element) which is a common 8 base pair palindromic sequence (5'-TATCGATA) required for the high level expression of replication related genes such as those encoding DNA polymerase α and cyclin A genes (Hirose et al., 1993; Yamaguchi et al., 1995a; Ohno et al., 1996). The DRE-DREF (DRE-binding factor) system appears to play a key role in the differentiation-coupled repression of cell proliferation during *Drosophila* embryogenesis (Hirose et al., 1994). Recently, we found that promoter region of the *D-raf* gene contains two sequences homologous to the DRE and demonstrated DRE/DREF involvement in regulation of the *D-raf* gene (Ryu et al., 1997). Whether expression of the *Drosophila* PCNA and *D-raf* genes is coordinately regulated in proliferation-controlled cells is very interesting.

UA, a pentacyclic triterpene acid, has been reported to possess antitumor activities including inhibition of skin tumorigenesis (Huang et al., 1994), induction

* To whom correspondence should be addressed.
Tel: 82-51-510-2278, Fax: 82-51-513-9258.

of tumor cell differentiation (Lee et al., 1994), antitumor promotion (Ohigashi et al., 1986), and antiinvasive activity (Cha et al., 1996).

To investigate whether expression of the PCNA and *D-raf* genes is coordinately regulated in proliferation-controlled cells, in this study we examined effects of UA on proliferation of the *Drosophila* Kc cells and on expression of the both genes. We report here inhibitory effects of UA on proliferation of the *Drosophila* Kc cells and on expression of the *Drosophila* PCNA and *D-raf* genes.

Materials and Methods

Plasmids

The plasmid p5'-168DPCNACAT contains the PCNA gene fragment spanning from -168 to +23 with respect to the transcription initiation site placed upstream of the CAT gene in the plasmid pSKCAT (Yamaguchi et al., 1991). The plasmid p5'-878DrafCAT contains the *D-raf* gene fragment spanning from -878 to +358 with respect to the transcription initiation site fused with the CAT in the plasmid pSKCAT (Ryu et al., 1997) (Fig. 1). pAct5C-CAT contains the *Drosophila* actin 5C promoter (-2500 to +88) fused with the CAT in the plasmid pSKCAT (Bond et al., 1986). The plasmid pSKCAT (parent vector for the plasmids p5'-168DPCNACAT and p5'-878DrafCAT) carry the polylinker region of Bluescript SK⁻ in front of the CAT-coding region, splicing signals of the simian virus 40 early gene, and poly (A) addition signal of simian virus 40 early gene. The plasmids p5'-168DPCNACAT and p5'-878DrafCAT were used as reporter plasmids and the plasmids pSKCAT and pAct5C-CAT were used as a negative and positive control, respectively. All plasmids were prepared

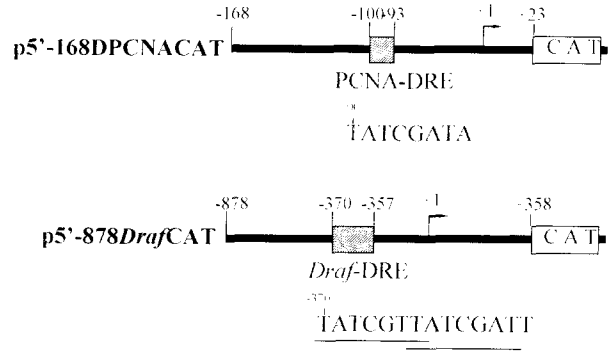

Fig. 1. Constructs of reporter plasmids p5'-168DPCNACAT and p5'-878DrafCAT. The plasmid p5'168DPCNACAT contains the PCNA gene fragment spanning from -168 to +23 with respect to the transcription initiation site placed upstream of the CAT gene in the plasmid pSKCAT. The plasmid p5'-878DrafCAT contains the *D-raf* gene fragment spanning from -878 to +358 with respect to the transcription initiation site fused with the CAT in the plasmid pSKCAT. The hatched boxes indicate the DRE sequence and the numbers with vertical lines indicate nucleotide positions with respect to the transcription initiation site.

through two cycles of ethidium bromide CsCl density gradient centrifugation.

Cell culture, DNA transfection, and CAT assay

Drosophila Kc cells (Echalier et al., 1970) were grown at 25℃ in M3 (BF) medium (Sigma) supplemented with 2% fetal bovine serum and 0.5% penicillin-streptomycin (GIBCO-BRL). 5×10^6 cells were plated onto 60 mm plastic dishes. 16 h later, cells were transfected with 10 μg of DNA by the calcium phosphate coprecipitation method described elsewhere (Di Nocera et al., 1983). Five hour later, cells were treated with UA (Sigma). Forty eight hour after transfection, cell extracts were prepared, and CAT activities were measured as described elsewhere (Yamaguchi et al., 1988). CAT activity was normalized to protein amounts determined by Bicinconinic acid (BCA) Protein Assay Reagent (PIERCE). The CAT activities were quantified by an imaging analyzer BAS1500 (Fuji Film).

DNA content assay by fluorometric method

DNA content assay was carried out with fluorometric method described by Erwin et al., (1981). Triplicate samples of 1.0×10^6 cells were dispensed in 60 mm culture dishes. Cells were counted by using 0.25% trypan blue (Sigma) and a hemocytometer (Buller and Dawson, 1992). After 16 h incubation, cells were treated with UA. After 48 h incubation, cells were washed with ice cold PBS, and then lysed in 5% trichloroacetic acid (TCA) on ice for 5 min. Cell extracts were removed from culture dishes with rubber policeman, and then stored at 4℃ for at least 4 h to ensure complete precipitation of acid insoluble materials. The precipitate obtained by centrifugation at 1000×g for 15 min was suspended by vortexing in 500 μl of 5% TCA. The suspension was hydrolysed for 30 min at 90℃ and cooled to 4℃. Solubilized aldehydes resulted from DNA hydrolysis were separated from the precipitate by centrifugation at 1000×g for 15 min. 100 μl of 3, 5-diaminobenzoic acid (DABA, 200 μg/μl stock solution) was added to the TCA supernatant. The reaction between DNA aldehydes and DABA was allowed to proceed for 1 h at 60℃. To terminate the reaction, 2 ml of 1 N HCl was added. The fluorescence of product was determined with a fluorescence spectrophotometer (Hitachi. F-4500) at absorption wave length 415 nm and emission wave length 504 nm. For standard samples, salmon sperm DNA (sodium salt), 1 mg/ml stock solution, was prepared in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and appropriate aliquots were dried at 60℃. Standards were prepared by the same procedures as mentioned above.

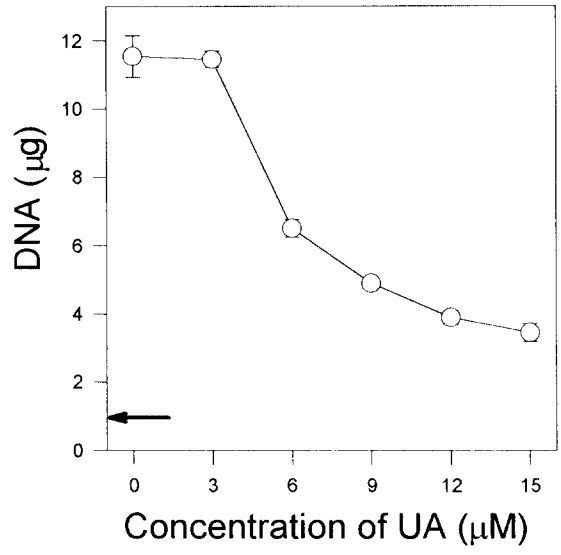

Fig. 2. Effect of UA on proliferation of the Kc cells shown by DNA content assay. To measure growth of the Kc cells, the amount of total DNA was measured with fluorometric method described by Erwin et al. (1981), using a computerized spectrofluorimeter. The values are the mean values of triplicate samples. Arrow represents the amount of DNA from cells just before UA-treatment.

[^{3}H]Thymidine incorporation assay

The [^{3}H]thymidine incorporation assay was carried out as described by Werner et al., (1985). Quadruplicate samples of 2×10^5 cells were dispensed in 24 well culture plate (Corning). After a 16 h culture, cells were treated with UA. After a 43 h culture, a 0.5 μCi of methyl tritiated thymidine (5.0 Ci/mmol, Amersham) was added to each well. After a 5 h culture, cells were washed three times with cold PBS and fixed with methanol for 30 min. The unincorporated [^{3}H]thymidine was removed by washing with 5% ice cold TCA. The radioactivity was measured by a liquid scintillation counter (LKB, 1219 RACKBETA).

Results

The kinetics of cell growth were analyzed by DNA contents of cultures and by the incorporation of [^{3}H]thymidine into DNA. The growth kinetics shown in DNA contents of the Kc cells treated with UA for 48 h are shown in Fig. 2. UA showed an inhibitory effect on proliferation of the Kc cells in a concentration dependent fashion. However, amounts of DNA of the cultures treated with the highest concentration (15 μM) of UA were more than those of incipient cultures (just before the chemical treatment) as shown in Fig. 2. Therefore, these results mean that UA inhibited proliferation of the Kc cells but not affected viability of the cells. The IC_{50} value of anti-proliferative effects of UA was determined to be about 7.5 μM in DNA content assays. Effects of UA on incorporation of [^{3}H]thymidine into DNA of the Kc cells are shown in Fig. 3. The incorporation of

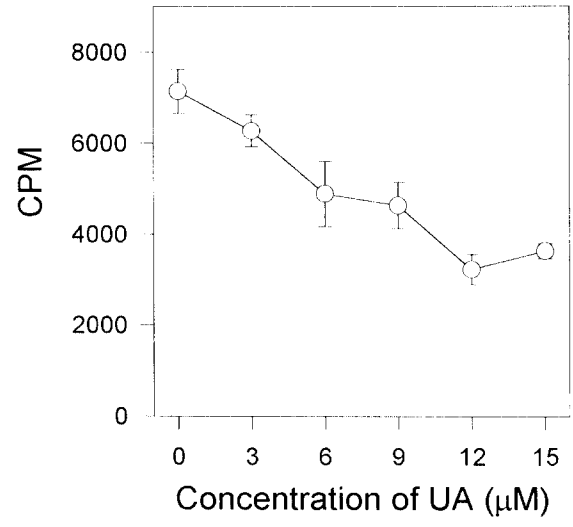

Fig. 3. Effect of UA on proliferation of the Kc cells shown by [^{3}H]thymidine incorporation assay. 2×10^5 cells were seeded in 24-well Corning multidishes. 16 h later, each concentration of UA was added into the cultures. The cultures were incubated for 48 h. For the final 5 h of the incubation, 0.5 μCi of methyl tritiated thymidine was added to each culture. Cells in each well were harvested and lysed. The radioactivity was measured with a liquid scintillation counter. The value are the mean value of quadruplicate cultures.

[^{3}H]thymidine into DNA was also inhibited in the UA-treated cells in a concentration-dependent fashion. These results obtained in the DNA content assays and the [^{3}H]thymidine incorporation assays indicate that UA have inhibitory effects on proliferation of the *Drosophila* cultured Kc cells.

Effects of UA on expression of the PCNA and *D-raf* genes were examined. For assaying expression of the PCNA and *D-raf* genes, the plasmid p5'-168 DPCNACAT and the plasmid p5'-878DrafCAT mentioned in Materials and Methods were used, respectively. Kc cells transfected with p5'-168DPCNACAT or p5'-878DrafCAT by the calcium phosphate method were cultured for 48 h in media without or with UA at various concentrations. CAT activities of extracts of the UA-treated (or untreated) cells transiently expressed the reporter plasmid p5'-168DPCNACAT or p5'-878DrafCAT (Fig. 4A and B) showed that UA reduced the expression of both PCNA and *D-raf* genes in a concentration-dependent manner. And, we detected that the CAT activities of the Kc cells transfected with pAct5C-CAT (used as a positive control) and treated with 12 μM of UA showed no change compared with that of the UA-untreated cells (data not shown). The extent of repression of the *D-raf* gene expression by UA was similar to that of the PCNA gene expression (Fig. 4C and D).

Discussion

Expression of genes for DNA replication enzymes is closely correlated with the proliferating state of cells and repressed in accordance with the progres-

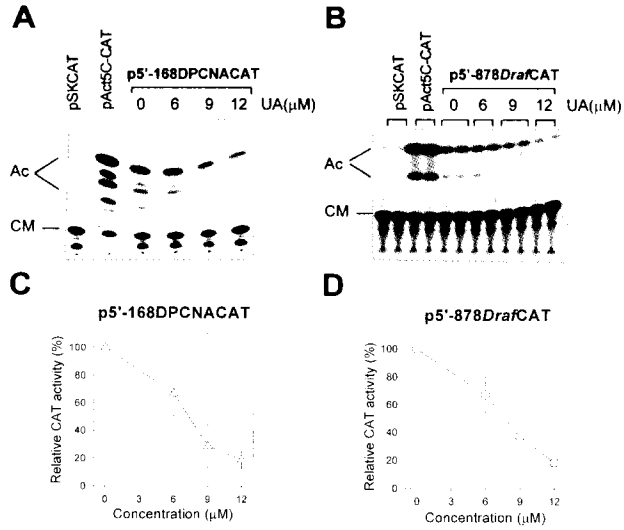

Fig. 4. Expression of the *Drosophila* PCNA and *D-raf* genes in the UA-treated or untreated Kc cells. 10 μg of reporter plasmids, p5'-168DPCNACAT (A), or p5'-878DrafCAT (B) was transfected into the Kc cells. The plasmids pSKCAT and pAct5C-CAT were used as a negative and positive control, respectively. Five hour after transfection, cells were treated with UA and 48 h after transfection, cell extracts were prepared and used for CAT assays (A and B). The CAT activities from spots corresponding to acetylated [^{14}C]chloramphenicols on the thin-layer plates were quantified by an imaging analyzer BAS1500 (Fuji Film) and plotted against activity in UA-untreated Kc cells (C and D). CAT activity was normalized to protein amounts determined by BCA assay. The relative values were averages of results from three independent experiments. Ac, acetylated forms of chloramphenicol; CM, non-acetylated forms of chloramphenicol.

sion of differentiation in various tissues during development (Matsukage et al., 1986; Matsukage et al., 1994). Mammalian genes such as these encoding DNA polymerase α, thymidine kinase, dihydrofolate reductase (DHFR) and PCNA contain the transcription factor E2F-binding site within their promoter regions or first intron (Nevins, 1992; Slansky et al., 1993; Yamaguchi et al., 1994; Lee et al., 1995). Expression of these genes increases dramatically at late G1 in response to growth stimulation (Nevins, 1992; Miyazawa et al., 1993). As in the case of mammalian, the *Drosophila* DNA polymerase α gene and PCNA gene also contain E2F recognition sites (Ohtani and Nevins 1994; Yamaguchi et al., 1995b) and these sites essentially function in the regulation of promoter activity of these genes during development (Yamaguchi et al., 1995b). Expression of these genes are also regulated by the DRE/DREF system (Hirose et al., 1993; Yamaguchi et al., 1995a). *D-raf* is required for the regulation of cell proliferation and differentiation.

UA effectively inhibited the proliferation of cultured Kc cells of *Drosophila* as shown in [^{3}H]thymidine incorporation assays and DNA content assays (Fig. 2 and 3). UA, which is a tumor cell differentiation-inducing agent (Lee et al., 1994), showed inhibitory effects on expression of the PCNA and *D-raf* genes in a concentration-dependent manner (Fig. 4A and B). The extent of repression of the *D-raf* gene expression by UA was similar to that of the PCNA gene expression (Fig. 4C and D). Expression of PCNA is known to be regulated by cell cycledependent transcription (Jaskulski et al., 1988; Liu et al., 1989). Little is known about the control of *raf* proto-oncogene expression. The repression of the *D-raf* gene expression by UA indicate that expression of the *D-raf* gene can undergo modulation in association with change in cellular proliferation.

Our results suggest that expression of the PCNA and *D-raf* genes is coordinately regulated in at least UA-treated Kc cells and that down-regulation of expression of PCNA and *D-raf* might be related with the antitumor activities of UA, although the detailed mechanism by which UA reduced the expression of both genes remains to be clarified.

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