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Roles of POLD4, smallest subunit of DNA polymerase δ, in nuclear structures and genomic stability of human cells

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ABSTRACT

Mammalian DNA polymerase δ (pol δ) is essential for DNA replication, though the functions of this smallest subunit of POLD4 have been elusive. We investigated pol δ activities *in vitro* and found that it was less active in the absence of POLD4, irrespective of the presence of the accessory protein PCNA. shRNA-mediated reduction of POLD4 resulted in a marked decrease in colony formation activity by Calu6, ACC-LC-319, and PC-10 cells. We also found that POLD4 reduction was associated with an increased population of karyomere-like cells, which may be an indication of DNA replication stress and/or DNA damage. The karyomere-like cells retained an ability to progress through the cell cycle, suggesting that POLD4 reduction induces modest genomic instability, while allowing cells to grow until DNA damage reaches an intolerant level. Our results indicate that POLD4 is required for the *in vitro* pol δ activity, and that it functions in cell proliferation and maintenance of genomic stability of human cells.

Introduction

Eukaryotic DNA polymerase δ (pol δ), a key enzyme that participates in DNA replication and repair, consists of four subunits; POLD1 (catalytic subunit, alternatively called p125), POLD2 (p50), POLD3 (p68), and POLD4 (p12) [1,2]. Among those, POLD4 binds to POLD1, POLD2, and an accessory protein of PCNA, which allows pol δ to exhibit its full activity [1,3].

A previous study showed that the *POLD4* ortholog of *Cdm1* in *Schizosaccharomyces pombe* is a non-essential gene related to cell growth, division, and sensitivity to DNA damaging reagents [4]. *Saccharomyces cerevisiae* does not have a POLD4 counterpart, indicating that POLD4 is dispensable in lower eukaryotic cells. In contrast, siRNA-mediated knockdown of POLD4 caused a significant decrease in the proliferation rate of FGF2-activated mouse-endothelial cells [5]. However, it remains unknown whether POLD4 is required for other types of mammalian cells, such as those related to human cancer, or if it has additional functions in mammalian cells.

In the present study, we analyzed the roles of POLD4 for cell proliferation in human lung cancer cell lines. Our findings indicate that POLD4 is required for maintaining the proper nuclear structures and suggest that the pathological structures reflect elevated DNA damage in chromosomes.

Materials and methods

Antibodies. The antibodies used in this study were anti-POLD4 (POLD4 subunit of pol δ) ascites (2B11, Abnova, Taipei City, Taiwan), anti-lamin B (c-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-γ-tubulin (Sigma–Aldrich, St. Louis, MO).

In vitro pol δ activity. Three- and 4-subunit DNA from pol δ were expressed in *Escherichia coli*, and purified as described previously [6]. pol δ activity was determined in a reaction mixture (25 μl) containing 20 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM each of dGTP, dATP, dCTP, and [α-^{32}P]dTTP, 100 ng poly dA-oligo dT (GE Healthcare, Piscataway, NJ), 86 ng (1.0 pmol as a trimer) of PCNA, and 11–88 ng (46–372 fmol) of pol δ at 30 °C for 10 min. Following incubation, the reactions were terminated with 2 μl of 300 mM EDTA. pol activity was determined with reference to the incorporation of [α-^{32}P]dTMP, as previously described [6].

Colony formation assay. To assess cell proliferation, colony formation assays were performed as previously described [7]. In order to rule out the off-target effect, we designed two independent DNA sequences as follows: MS543F, 5′-GATCCCCagtctctggcatctctatcATTCAAGAGATgatagagatgccagagactTTTTTGGAAA-3; MS544R, 5′-AGCTTTTCCAAAAAagtctctggcatctctatcATCTCTTGAATgatagagatgccagagactGGG-3; MS551F, 5′-GATCCCCgcatctctatcccctatgaATTCAAGAGATtcataggggatagagatgcTTTTTGGAAA-3; and MS552R, 5′-AGCTTTTCCAAAAAgcatctctatcccctatgaATCTCTTGAATtcataggggatagagatgcGGG-3, in which the targeting sequences are indicated in lowercase letters. To construct shRNA vectors, MS543F and MS544R

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(polD4-5), and MS551F and MS552R (polD4-3-1) were annealed, then inserted between the restriction sites BglII and HindIII in PH1RNAneo [7]. Cells transfected with a vector carrying either polD4-3-1 or polD4-5 were cultured in media containing 1 mg/ml G418 (Invitrogen, Carlsbad, CA 10131-027), which was reduced by 0.2 mg/ml every 2 days until it reached 0.4 mg/ml. When colonies grew to visible seizes, they were fixed by cold methanol for 5 min and stained with 4% Giemsa for 15 min at room temperature.

RNA interference. Transfection was carried out using 50 nmol/L of a small interfering RNA (siRNA) duplex (Sigma–Aldrich) targeting each mRNA, or negative control 1# (Ambion) with Lipofectamine-2000 (Invitrogen). The sequences of si*POLD4* were the same as those of polD4-3-1: *POLD4* (si*D4*) sense, 5′-GCAUCUCUAUCCCCUAUGATT-3′; and antisense, 5′-UCAUAGGGGAUAGAGAUGCTT.

Laser scanning cytometry (LSC). Following an overnight culture, 3×10^5/ml Calu6 cells on coverslips were fixed by cold methanol, washed with PBS, and incubated with 1 mg/ml RNase A in 50 mM Tris–HCl, pH 7.5, at 37 °C for 1 h. Cells were further treated with 50 μg/ml propidium iodide in a mixture containing 180 mM Tris–HCl, pH 7.5, 180 mM NaCl, and 70 mM $MgCl_2$ for 15 min. Nuclei structures and DNA contents were analyzed using a Laser Scanning Cytometer (LSC, Olympus, Tokyo, Japan), with DNA content at the G1 peak regarded as 2N, though Calu6 cells carry a greater amount of DNA chromatin than normal cells.

Cell cycle synchronization. Calu6 cells were synchronized according to the method of Nakagawa et al. [8], with minor modifications. In brief, 24-h treatment with 2 mM thymidine was used to arrest exponentially proliferating cells in the S phase. Cells were then released from arrest by three washes in PBS and grown in fresh medium for 15 h, then 1 μM of aphidicolin was used for the second block for 10 h. After releasing by three washes in PBS, cells were incubated in RPMI1640 containing 5% fetal bovine serum and harvested at various time points.

Immunofluorescence. Following an overnight culture, 3×10^5/ml Calu6 cells on coverslips were transfected with siRNA as described above. After 48 h, they were fixed in 4% paraformaldehyde for 10 min at room temperature, followed by treatment with cold methanol for 2 min. The coverslips were washed three times in PBS, treated with PBS containing 0.25% Triton X-100 on ice for 30 min, and incubated with anti-lamin B or anti-γ-tubulin antibody overnight at 4 °C. The cells were then washed three times in PBS, incubated for 1 h with Alexa 488-conjugated secondary antibody (Molecular Probes, OR, USA), and analyzed using an Olympus BX60 (Olympus).

Results and discussion

DNA synthesis activities of pol δ with or without POLD4 in vitro

In order to analyze POLD4 functions related to intrinsic pol δ activity, 3- and 4-subunit structures of pol δ were expressed and purified. In the absence of POLD4, pol δ was less active than the holoenzyme in a reaction containing poly dA–dT as a template primer (Fig. 1A), with a similar result obtained when the accessory protein of PCNA was omitted from the reaction (Fig. 1B). These results are consistent with those of previous studies [1,3] and indicate that POLD4 is required for pol δ to exhibit its full catalytic activity.

POLD4 required for cell proliferation

A previous genetic study of *S. pombe* demonstrated that the *POLD4* ortholog of *Cdm1* is a non-essential gene for cell growth,

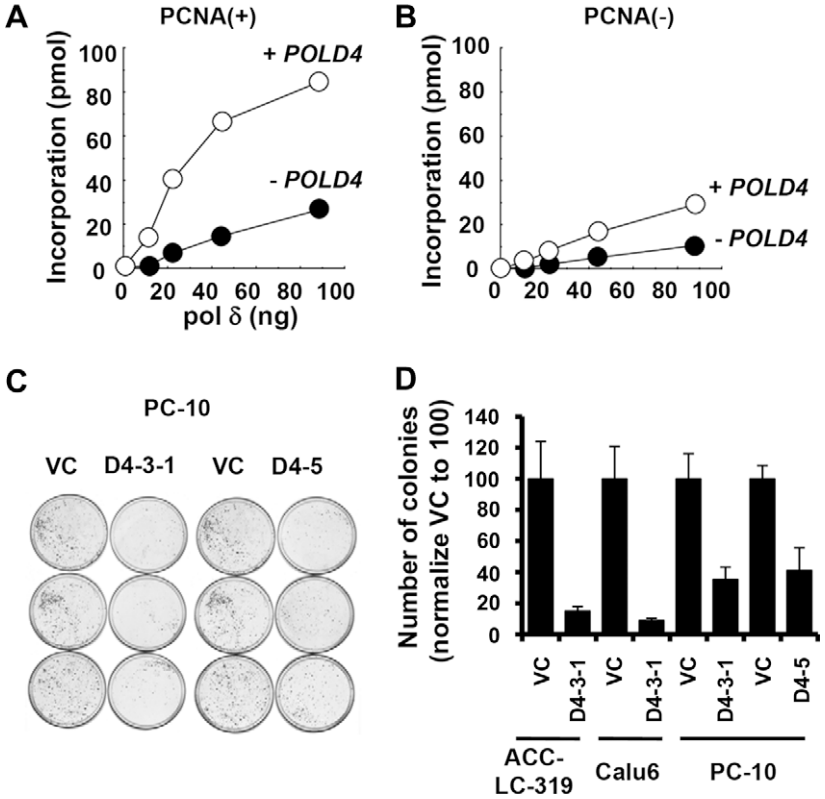

Fig. 1. *In vitro* DNA synthesis activities of pol δ and effect of POLD4 depletion on colony formation activity. (A) pol δ activities were measured and plotted as described in Materials and methods. (B) The same reactions were carried out in the absence of PCNA. (C) PC-10 was used for transfection with plasmids carrying either D4-3-1 or D4-5, and colony formation activity was determined as described in Materials and methods. VC represents vector control. (D) Results of the colony formation assay were plotted in a graph.