Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1373–1380
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Mechanism of cell cycle arrest by sulfoquinovosyl monoacylglycerol with a C18-saturated fatty acid (C18-SQMG)

Chikako Murakami[a], Takeshi Miuzno[b], Fumio Hanaoka[b,c], Hiromi Yoshida[a,d], Kengo Sakaguchi[e], Yoshiyuki Mizushina[a,d,*]

[a]*Laboratory of Food & Nutritional Science, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan*
[b]*Cellular Physiology Laboratory, RIKEN (The Institute of Physical and Chemical Research), and CREST, Japan Science and Technology Corporation, Wako, Saitama 351-0198, Japan*
[c]*Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan*
[d]*High Technology Research Center, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan*
[e]*Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Noda, Chiba 278-8510, Japan*

Received 24 September 2003; accepted 5 December 2003

Abstract

We have screened the inhibitors of mammalian DNA polymerases from natural products, and in the process found that either sulfoglycolipids or sulfoquinovosyl monoacylglycerol with a C18-saturated fatty acid (C18-SQMG), potently and selectively inhibited the activity of mammalian DNA polymerase (pol) ε and moderately the pol α [Biochem. J. 370 (2003) 299]. C18-SQMG was a cancer cell growth suppressor and a promissive anti-tumor agent [Jpn. J. Cancer Res. 93 (2002) 85]. The purpose of this study was to elucidate the cell growth inhibition mechanism of C18-SQMG using HeLa cells. Analyses of the cell cycle and cyclin expression suggested that C18-SQMG arrested the cell cycle at intra-S phase, and the inhibition manner of DNA replication by C18-SQMG was similar to that by hydroxyurea. However, the DNA replication block by C18-SQMG did not induce degradation of Cdc25A protein, which was required for the replication block by hydroxyurea. C18-SQMG somewhat delayed mitosis because it induced phosphorylation of protein kinases, such as checkpoint kinases 1 and 2. These results suggest that C18-SQMG at first blocked DNA replication at the S phase by inhibiting replicative DNA polymerases, such as α, and then as the result of the inhibition, the other checkpoint signals associated with the pol ε might have responded.

Keywords: C18-SQMG; Hydroxyurea; DNA polymerases α and ε; Cyclin; Chk1; Chk2

1. Introduction

According to recent studies, eukaryotic DNA replication complex comprises at least origin recognition complex (ORC) proteins, including ORC-1 to ORC-6, mini-chromosomal maintenance (MCM) proteins (MCM-2 to MCM-7), CDC6, CDT1, CDC45, Cdc7-Dbf4 kinase complex, and DNA polymerases, such as α, δ, and ε (pol α, δ, and ε) [1–4]. In addition, DNA helicase (MCM), proliferating cell nuclear antigen (PCNA) and topoisomerases work in concert with the complex [1–4]. DNA helicase and topoisomerases were suggested to have roles in loosening the distortion of double-stranded DNA. PCNA, a ring-shaped homotrimeric protein, is recruited onto the DNA by a multiprotein clamp loader, replication factor C (RFC), which couples the hydrolysis of ATP with the opening and closing of the PCNA ring around the DNA. The replicative pol δ, then connects with the PCNA and carries out processive synthesis of both the leading and lagging DNA strands [5,6]. In the cell biological study, DNA replication required the recruitment of multiple components during the S phase of the cell cycle. The important components are replicative pol α, δ, and ε, essential for the replication of the eukaryotic genome as shown by genetic studies in budding yeast [7,8]. Among them, pol α-primase

* Corresponding author. Tel.: +81-78-974-1551x3232; fax: +81-78-974-5689.
E-mail address: mizushin@nutr.kobegakuin.ac.jp (Y. Mizushina).

Abbreviations: C18-SQMG, sulfoquinovosyl monoacylglycerol with a C18-saturated fatty acid; HU, hydroxyurea; pol, DNA polymerase; ORC, origin recognition complex; MCM protein, mini-chromosomal maintenance protein; PCNA, proliferating cell nuclear antigen; ATM, ataxia telangiecstasia mutated; ATR, ATM- and Rad3-related protein kinase; Chk, checkpoint kinase; CDK, cyclin-dependent kinase.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.12.004

is the only enzyme that can start DNA synthesis *de novo*. In addition, the second largest subunit of pol α, B-subunit, takes on a cell cycle-regulatory role [9]. In budding yeast, unphosphorylated forms of B-subunit accumulate only in G1-arrested cells, and this phosphorylation appears to correlate with CDC6-independent pol α loading onto the chromatin fraction [10]. Since C18-SQMG is obviously an inhibitor of replicative polymerases, such as pol α, δ, and ε [11], the action must be related to the system of the DNA synthesis *de novo*, and the genomic integrity of the cell.

We tried to find inhibitors of mammalian DNA polymerases from natural products, and consequently successfully isolated many novel compounds. In the novel compounds, some sulfoglycolipids were inhibitors which potently and selectively inhibit the activities of mammalian DNA polymerases, especially replicative polymerases [12–15]. One of them, C18-SQMG, effectively inhibited cancer cell proliferation by arresting the cell cycle at the G1/S phase, and finally induced apoptosis [16]. The compound was developed as a clinically promissive antitumor agent [17]. The molecular action mechanism subsequently proposed was that the G1 arrest and apoptosis by C18-SQMG was induced as a result of inhibition of the DNA polymerases, and the arrest was mediated by the p53-p21-pRb pathway [18]. The *in vitro* studies using cultured cells have shown that as a result of the inhibition of the replicative polymerases, C18-SQMG induces the replication block in the cell cycle [16,18]. However, it is not yet clear how C18-SQMG prevents the progression of DNA replication at the S phase, or whether the inhibition of the DNA polymerases is involved in the replication block. We tried to answer these questions in this study.

In our previous preliminary studies of the inhibitory action of C18-SQMG, we showed that the compound may destroy the genomic integrity of the cell [16,18]. To maintain genomic integrity, eukaryotic cells have checkpoint mechanisms which are activated by damaged DNA or unreplicated DNA, leading to cell cycle arrest, DNA repair, and cell death [19]. In eukaryotes, upstream elements of the checkpoint signaling pathways include the kinase ataxia telangiecstasia mutated (ATM), a member of the phosphatidylinositol 3-kinase family, and its relative ATM- and Rad3-related protein kinase (ATR) [20–23]. ATM and ATR phosphorylate and activate the effector kinases Cds1 (homologue of checkpoint kinase (Chk) 2) and Chk1, respectively, which in turn block cyclin-dependent kinase (CDK) activity. It was revealed that Chk1 regulates the stability of Cdc25A [24]. Cdc25A is apparently important during initiation and progression to the S phase [25]. In mammalian cells, Cdc25A was usually activated with the ATR-Chk1 pathway in the DNA replication block [26]. Thus, the DNA replication block activates Chk1, resulting in rapid Cdc25A degradation and intra-S phase arrest. Since SQMG inhibited replicative DNA polymerase, it was suggested that SQMG perturbed the cell cycle involved in the replication checkpoint response followed by inhibition of replicative DNA polymerases. Here, to confirm this suggestion, we investigated details of the mechanisms that required intra-S phase arrest in response to SQMG in human cancer cells. As observed in this study, C18-SQMG was not related to the degradation of Cdc25A protein, and it was suggested that it induced phosphorylation of Chk1 and Chk2, suggesting that C18-SQMG acted as the cell cycle inhibitor, indirectly working as a result of replicative polymerases, such as pol α and ε, but not being a replication-direct inhibitor.

2. Materials and methods

2.1. Antibodies

Rabbit polyclonal antibodies to cyclins A, B, and Cdc25B, and mouse monoclonal antibodies to Cdc25A, Cdc25C, Chk2, and pol ε were purchased from Lab Vision. Rabbit polyclonal antibody to Chk1 was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody to PCNA was purchased from Oncogene Research Products. Mouse monoclonal antibody to p180 catalytic subunit and p68 subunit of pol α was prepared as described previously [27].

2.2. Cell culture and cell viability assay

HeLa cells obtained from Health Science Research Bank were maintained in MEM medium supplemented with 10% fetal bovine serum at 37° (5% CO_2) in a humidified atmosphere. For the cell viability assay, cells were plated at 5×10^3 cells into each well of a 96-well microplate, and then SQMG was added at various concentrations. The cell viability was determined by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.

2.3. Cell cycle analysis

The cells (3×10^5 cells in a 35-mm dish) were collected by trypsinization and washed with ice-cold PBS *via* centrifugation. The cells were suspended in PBS, fixed with 70% ethanol (v/v), and stored at −20°. The cells were collected by centrifugation and stained with DAPI (2 mg/mL) for at least 20 min at room temperature in the dark. The DNA content was analyzed using a cell counter analyzer (Partec, CCA model) with Multicycle 3.11 software (Phoenix Flow Systems). The cell debris and fixation artifacts were gated out.

2.4. Western blot analysis

The cell extracts were prepared as described previously [28,29]. Briefly, cells were collected and washed in cold PBS. Proteins were then extracted with cold 0.1% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 100 mM

NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF) for 20 min at 4°. Triton-insoluble fractions were extracted in lysis buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS, 1 mM PMSF, and 1 mM leupeptin) by sonication, then pelleted by 16.000*g* for 10 min at 4°. The supernatant (nuclear extract) was analyzed by SDS–PAGE. To extract the protein binding chromatin, Triton-insoluble fractions were re-extracted by incubation in CSK buffer with 0.3 M KCl and collected *via* centrifugation at 3000 rpm for 10 min at 4° (chromatin-unbound fraction). The final pellet fraction (containing chromatin-bound proteins) was solubilized in CSK buffer with DNase I (0.5 mg/mL DNase I) for 30 min. The samples were centrifuged at 15,000 rpm for 15 min to obtain supernatant. The lysates were fractionated on SDS–PAGE, then blotted on a PVDF membrane. The blots were subsequently incubated with desired primary antibodies. After rinsing, the membranes were incubated with horseradish peroxidase-linked secondary antibody. The proteins were then detected with an enhanced chemiluminescence detection system (PerkinElmer Life Science, Inc.). Zero-D scan (version 1.0, M & S Instruments Trading Inc.) was used for densitometric quantitation.

3. Results

3.1. Molecular changes associated with the cell cycle effects of C18-SQMG

We first investigated whether C18-SQMG affected the cell cycle distribution of C18-SQMG-treated cells. When HeLa cells were treated with various concentrations of C18-SQMG for 24 hr, C18-SQMG inhibited the cell growth dose dependently (Fig. 1). Since the LD_{50} value was about 150 μM, the cells were treated with 150 μM C18-SQMG in the latter part of this experiments. Flow cytometry analysis showed that 24-hr treatment with C18-SQMG perturbed the cell cycle (Fig. 2A). The population of the cells at the S phase decreased from 23.6 to 17.5% (upper and middle panels in Fig. 2A). This decrease occurred by increasing the G1 cell population, and consequently the population of the cells at the G1 phase increased from 58.2 to 73.1% (upper and middle panels in Fig. 2A). Interestingly, the population of the cells at the

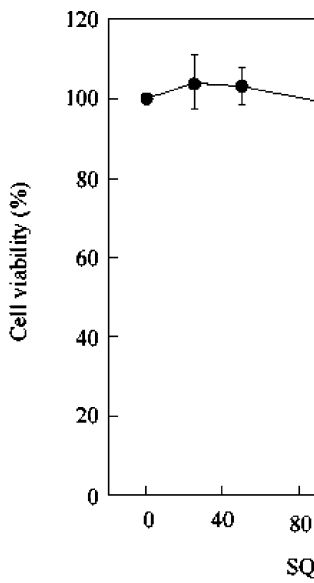

Fig. 1. Effect of sulfoquinovosyl monoacylglycerol (SQMG) on the proliferation of HeLa cells. Dose-dependent growth inhibition of HeLa cells incubated with various concentrations of SQMG for 24 hr. Cell proliferation was determined by MTT assay. Values are shown as means ± SEM for four independent experiments.

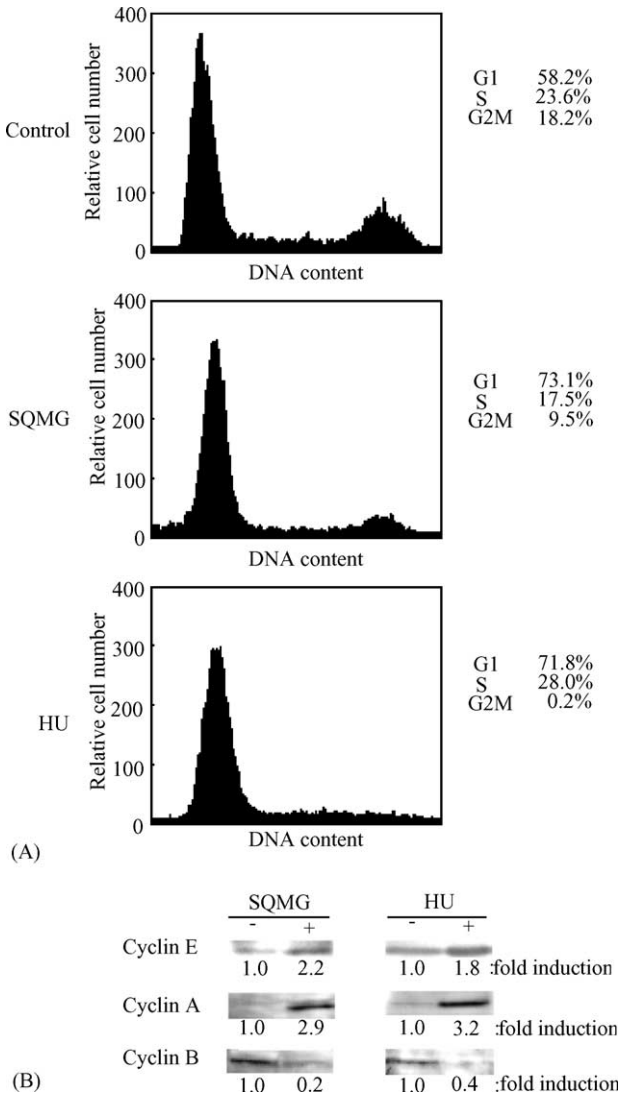

Fig. 2. Effect of sulfoquinovosyl monoacylglycerol (SQMG) on cell cycle. (A) Flow cytometric analysis of HeLa cells were treated with 150 μM SQMG or 2 mM HU for 24 hr. The cell cycle distribution was calculated as the percentage of cells containing G1, S, and G2/M phase. (B) Cyclin expressions were analyzed by Western blotting. Cell extracts of the nuclear fraction were prepared from cells treated with 150 μM SQMG 2 mM HU for 24 hr. Cyclins E, A, and B were detected with specific antibodies. Densitometric analysis of the proteins was performed and fold induction was calculated.

G2/M phase also decreased from 18.2 to 9.5%. This suggests that C18-SQMG related the cell cycle-associated signal mechanism, since C18-SQMG never directly bound to DNA and chromatin as described previously [11].

Therefore, next we examined whether C18-SQMG had an effect on to the cell cycle-related proteins, cyclins A, B, and E. As shown in Fig. 2B, C18-SQMG was associated with modified expression of cyclins by Western blotting. Cyclins A and E proteins significantly increased after cell treatment of C18-SQMG, but the expression of cyclin B protein was suppressed. Cyclin E protein is present from the end of the G1 phase to the early S phase, and cyclin A protein keeps accumulating through the G1 to the S phase [30,31]. It was suggested that cyclins A and E link the transition mechanism from the G1 to the S phase, and appear when the cells start to synthesize DNA [30,31]. On the other hand, cyclin B was associated with the mechanism of G2/M transition [31]. It was suggested that C18-SQMG induced the replication block in the cells based on the above results. This phenomenon appeared to be very similar to the action of ribonucleotide reductase inhibitor hydroxyurea (HU), which was known to cause replication block. As shown in Fig. 2B, the cyclin expression patterns were very similar between C18-SQMG and HU, so for comparison we also analyzed the cell cycle and the cyclin expression with HU. HU increased the number of the cells at the G1 phase from 58.2 to 71.8%, and the cells at the G2/M phase were mostly lost (18.2–0.2%) during 24-hr treatment (lower panel in Fig. 2A). Cyclin analysis in the cells treated with HU showed the same pattern as SQMG. This result supported the suggestion that SQMG induced replication block in cultured cells. Since in the presence of C18-SQMG a large amount of cells at the G2/M phase were detected, although some were lost (middle panel in Fig. 2A), the action might be different from the HU effect.

3.2. C18-SQMG-induced accumulation of Cdc25A

HU is also known to activate the proteins of Cdc25 and Chk. To study the mechanism of the C18-SQMG-induced replication block further in comparison with HU, Cdc25 and Chk protein expressions were determined by Western blotting (Fig. 3A and B). In humans, there are three Cdc25 homologues, Cdc25A, Cdc25B, and Cdc25C [32–35]. Cdc25A is involved in the intra-S phase checkpoint, and the level reaches a maximum at the end of the G1 phase and the early S phase [34]. Cdc25C promotes the G2/M transition through the dephosphorylation of Cdc2, and Cdc25B was proposed to act as an initiator of the same process [36–42]. As shown in Fig. 3A, although Cdc25A was rapidly degraded by HU, such Cdc25A degradation was not observed using the C18-SQMG treatment. In the presence of C18-SQMG, Cdc25B in the cells decreased, but Cdc25C expression remained unchanged through out the cell cycle. Therefore, the G1/S transition arrest induced by C18-SQMG showed no relation to the Cdc25A degradation. It is necessary to search upstream from Cdc25A in the intra-S phase checkpoint, especially Chk.

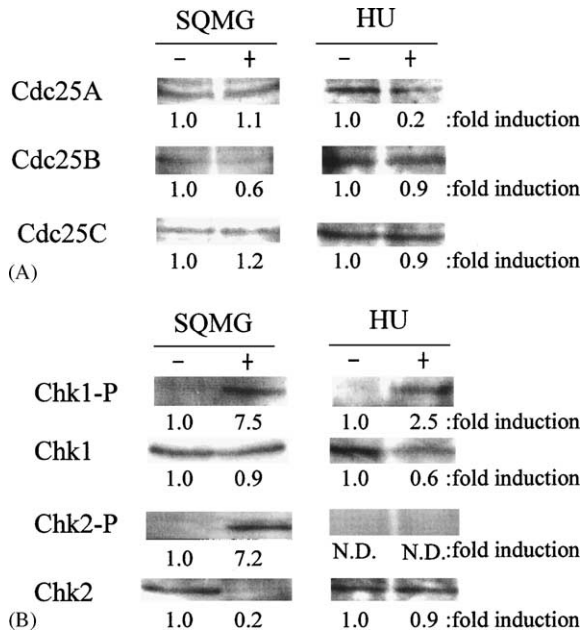

Fig. 3. Effect of sulfoquinovosyl monoacylglycerol (SQMG) on Cdc25A-independent intra-S phase arrest. Extracts of the nuclear fraction were prepared from cells with 150 μM SQMG or 2 mM HU treatment for 24 hr. (A) Cdc25A, Cdc25B, and Cdc25C were detected by Western blotting with specific antibodies. (B) Chk1 and Chk2 were detected with specific antibodies. The upper panels represent the phosphorylated form, and the lower panels represent the dephosphorylated form. Densitometric analysis of the proteins was performed and fold induction was calculated.

The current concept of a cell cycle checkpoint response places the regulatory kinase Chk1 upstream from Cdc25A in the intra-S phase checkpoint [43]. In fission yeast *S. pombe*, the DNA replication checkpoints are regulated by Chk1 and Cds1, homologue of Chk2 [44–46]. We therefore determined whether these kinases were activated when the intra-S phase checkpoint was activated by C18-SQMG. When the cells were treated with C18-SQMG, Chk1 and Chk2 were clearly phosphorylated (Fig. 3B). On the other hand, Chk1 was phosphorylated by HU, and phosphorylated Chk2 could not be detected in our experiment conditions (Fig. 3B). When the cells were exposed to ionizing radiation or UV light, they halted at the intra-S phase following the Cdc25A degradation, but the halted mechanism was p53 independent [47]. However, p53 triggered intra-S phase arrest that was in part induced by SQMG. Taken together, C18-SQMG may not be able to induce the phosphorylation of Chk1 protein sufficiently enough to degrade Cdc25A or the Chk1 signal used by other substrates besides Cdc25A. The intra-S phase arrest by C18-SQMG must occur through different mechanisms by HU.

3.3. Replicative DNA polymerases loading to chromatin

To confirm the involvement of C18-SQMG in DNA replication and the checkpoint system, we tried to detect

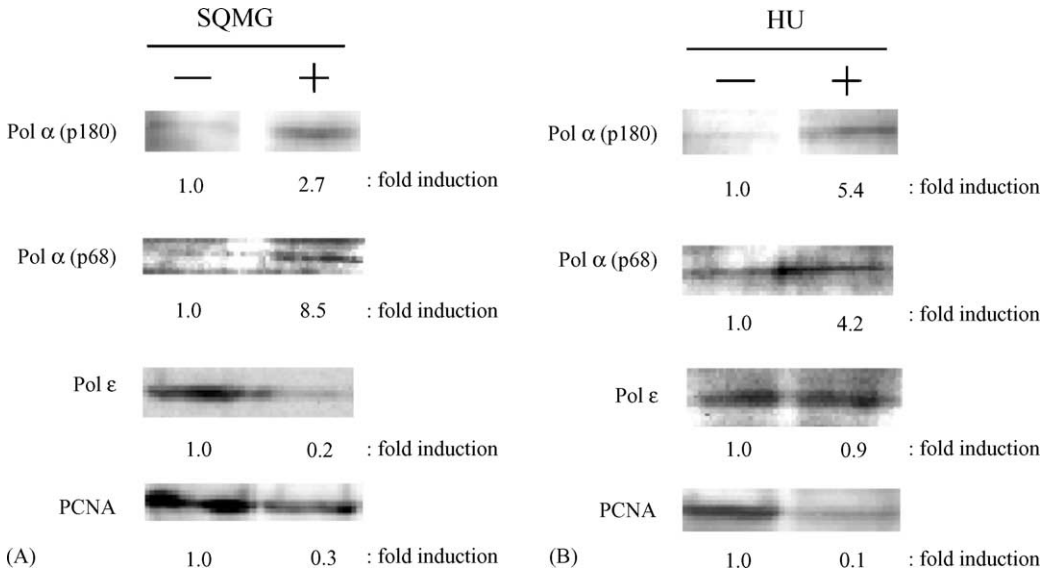

Fig. 4. Effect of sulfoquinovosyl monoacylglycerol (SQMG) on binding of DNA polymerase complex to chromatin. Western blot analysis of pol α p180 and p68 subunit, pol ε, and PCNA in the chromatin-bound fraction. (A) Extracts were prepared from cells treated with 150 μM SQMG for 24 hr. (B) Extracts were prepared from cells treated with 2 mM HU for 24 hr. Densitometric analysis of the proteins was performed and the fold induction was calculated.

two subunits of pol α, pol ε, and PCNA in the chromatin by Western blotting, because the primary molecular targets of C18-SQMG must be the replicative polymerases [11]. These proteins could play roles in DNA replication and the checkpoint system after they bind to the chromatin. Thus, we extracted the chromatin-bound fraction with DNase I treatment. When the cells were treated with C18-SQMG for 24 hr, both the p180 catalytic subunit and p68 subunits of pol α accumulated in the chromatin-bound fraction (Fig. 4A). However, PCNA and pol ε were dissociated from the chromatin after C18-SQMG-treatment (Fig. 4A). In the chromatin of the HU-treated cells, accumulation of the pol α subunits and the dissociation of PCNA were also observed. pol ε was detected in chromatin from the cells irrespective of the presence or absence of HU (Fig. 4B). These results using C18-SQMG and HU suggest that after the treatment of the inhibitors, pol α probably forms a stable complex with the primase in the chromatin, and pol ε itself might function as the signal because C18-SQMG can directly bind to the polymerase but HU cannot. The statement from the three dimensionally distorted pol ε protein might be associated with the signals in the DNA replication arrest.

4. Discussion

In our previous study [11], we demonstrated that a sulfoglycolipid, C18-SQMG, inhibited mammalian DNA polymerase activity, especially replicative polymerases. The C18-SQMG-treated cells induced replication arrest and apoptosis in a p53-dependent manner [16]. In this study, we investigated whether inhibition of the replicative DNA polymerases by C18-SQMG is involved in the replication arrest in the replication checkpoint response. Cell cycle analysis showed that C18-SQMG-treated cells reduced the number of the S phase and increased the number of the G1 phase (Fig. 2). C18-SQMG-induced cell cycle arrest contained the G2/M phase cells. Analysis of cyclins showed that C18-SQMG accumulated cyclins E and A, but reduced cyclin B expression (Fig. 2B). These results suggest that C18-SQMG induced predominant replication arrest and partly premature mitosis. In consideration of our previous suggestions [16,18], the phenomenon appeared to be very similar to the action mode of HU, and studying the phenomenon in comparison with the HU-induced effect may be valuable.

The HU-induced replication arrest caused Cdc25A degradation for rapid activation of Chk1 [43,48,49]. However, the C18-SQMG-treated cells accumulated Cdc25A. On the other hand, like another property of the inhibition by HU, C18-SQMG influenced Chk1 and Chk2, which act to elicit appropriate responses, such as cell cycle arrest. The common biochemical basis for the inhibition might be in the disturbance of the system mediated by Chk1 and Chk2. Since the replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Chk1, the similarity of the action modes between C18-SQMG and HU probably depends on the upstream signal for Cdc25A. Chk1 is essential in analogous checkpoint processes in mammalian cells to stall replication forks and suppress origin firing [50]. In yeast, the replication checkpoint functions depend on the Chk2 homologues Cds1 and Rad53 [51,52]. C18-SQMG-induced replication arrest might therefore be mediated directly by inhibiting replicative DNA polymerases themselves. One or more replicative polymerases might function as signal proteins for the Chk1 and Chk2 system.

To confirm this idea, we tested if pol α, pol ε, and PCNA bind to the chromatin immediate after treatment of C18-SQMG or HU. If pol α, pol ε, and PCNA functioned as the signal proteins, they would at first have to bind to the chromatin. Moreover, the reason is that as the action of C18-SQMG or HU, the DNA replication checkpoint may destroy genome stability. Consequently, in the cells treated with C18-SQMG, pol α accumulated into the chromatin, and PCNA and pol ε dissociated from the chromatin. On the other hand, in the HU-treated cells, only the accumulation of pol α and ε was observed. In fission yeast, the HU-induced cell cycle arrest and the Cds1-mediated checkpoint response were suggested to prevent the initiation complexes of the late-firing replicons, termed the intra-S phase checkpoint [53]. Moreover, for the HU-induced arrest, a stable pol α-primase complex is required to generate a signal for the activation of the intra-S phase checkpoint [53]. The perturbation of S phase initiation could be induced by different checkpoint responses in fission yeast [54]: following an early S phase stall caused by HU, a stable and intact pol α-primase complex is required to signal high levels of Cds1 kinase activation to prevent progression of the early replication fork and premature initiation of the late-firing replicons, and once DNA replication is initiated the synthesis of an initiation DNA structure by pol α is required to generate the S phase checkpoint to prevent inappropriate mitotic entry. In this model, C18-SQMG must influence the intra-S phase checkpoint predominantly through the inhibition of pol α activity. Since Chk1 is partially associated at the S/M checkpoint, C18-SQMG must also have a role at the S/M checkpoint.

As similarly described in Section 3, the statement from the three-dimensionally distorted pol ε protein might be associated with the signals in the DNA replication arrest. pol ε is also known to take part in the DNA replication, and pol ε may be the best candidate as a sensor involved in the replication-associated repair that either precedes or follows replication forks in early S phase [55]. Moreover, pol ε is not only a key regulator in DNA replication and repair, but is also important in monitoring the genetic statement in DNA replication events. Reportedly, a pol ε mutant viable but sensitive to DNA-damaged agents shows a cell cycle delay, and requires that the DNA-damaged checkpoint maintain cell viability [56]. In addition, the ATR signal responds to the stalling of replication forks and generating single-stranded DNA phosphorylate Chk1/2 and other substrates, such as p53, Brca1, and SMC1, which participate in multiple checkpoint responses [57–62]. The pol ε inhibition caused by C18-SQMG must accumulate unreplicated DNA regions at the S phase. The regions may be recognized as damaged DNA and subsequently digested. Therefore, the inhibition of pol ε would also be attributable to the S/M checkpoint. In the process, the inhibition of pol α by C18-SQMG induces the intra-S phase checkpoint mediated by Chk1, and the S/M checkpoint response mediated by Chk1 and Chk2 resulting from the inhibition of pol ε by C18-SQMG as depicted in Fig. 5.

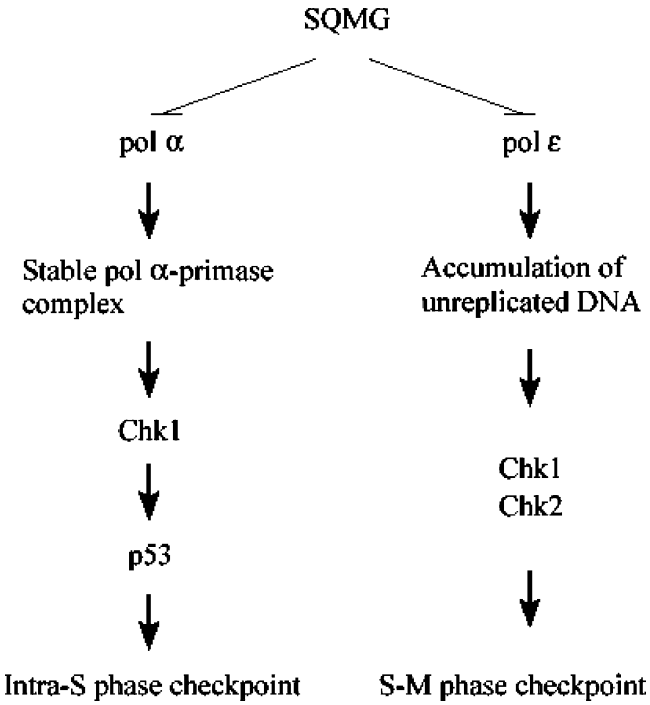

Fig. 5. The proposed response to sulfoquinovosyl monoacylglycerol (SQMG). Pol α inhibition induces replication block by intra-S phase checkpoint *via* Chk1, and pol ε dissociation from chromatin and inhibition of activity induces damaged DNA accumulation, and subsequently induces mitosis delay by S/M checkpoint *via* Chk1 and Chk2.

Acknowledgments

This work was partly supported by a Grant-in-Aid for Kobe-Gakuin University Joint Research (B) (C.M., H.Y., and Y.M.). Y.M. acknowledges a Grant-in-Aid from the Tokyo Biochemical Research Foundation, Sasakawa Grants for Science Fellows (SGSF) from the Japan Science Society and Grant-in-Aid 14780466 for Scientific Research, The Ministry of Education, Science, Sports and Culture, Japan.

References

[1] Waga S, Stillman B. The DNA replication fork in eukaryotic cells. Annu Rev Biochem 1998;67:721–51.
[2] Kelly J, Brown G. Regulation of chromosome replication. Annu Rev Biochem 2000;69:829–80.
[3] Hübscher U, Maga G, Spadari S. Eukaryotic DNA polymerases. Annu Rev Biochem 2002;71:133–63.
[4] Frouin I, Montecucco A, Spadari S, Maga G. DNA replication: a complex matter. EMBO Rep 2003;4:666–70.
[5] Prelich G, Kostura M, Marshak DR, Matthews MB, Stillman B. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature 1987;326:471–5.

[6] Tan CK, Castillo C, So AG, Downey KM. An auxiliary protein for DNA polymerase δ from fetal calf thymus. J Biol Chem 1986;261: 12310–6.
[7] Sugino A. Yeast DNA polymerases and their role at the replication fork. Trends Biochem Sci 1995;20:319–23.
[8] Hübscher U, Nasheuer HP, Syväoja JE. Eukaryotic DNA polymerases, a growing family. Trends Biochem Sci 2000;25:143–7.
[9] Mizuno T, Ito N, Yokoi M, Kobayashi A, Tamai K, Miyazawa H, Hanaoka F. The second largest subunit of the mouse DNA polymerase α-primase complex facilitates both production and nuclear translocation of the catalytic subunit of DNA polymerase α. Mol Cell Biol 1998;18:3552–62.
[10] Desobuets C, Santocanale C, Drury L, Perkins G, Foiani M, Plevani P, Diffley J. Evidence for a Cdc6p-independent mitotic resetting event involving DNA polymerase α. EMBO J 1998;17:4139–46.
[11] Mizushina Y, Xu X, Asahara H, Takeuchi R, Oshige M, Shimazaki N, Takemura M, Yamaguchi T, Kuroda K, Linn S, Yoshida H, Koiwai O, Saneyoshi M, Sugawara F, Sakaguchi K. A sulphoquinovosyl diacylglycerol is a DNA polymerase ε inhibitor. Biochem J 2003;370:299–305.
[12] Sahara H, Ishikawa M, Takahashi N, Ohtani S, Sato N, Gasa S, Akino T, Kikuchi K. In vivo anti-tumour effect 3′-sulphoquinovosyl 1′-monoacylglyceride isolated from sea urchin. Br J Cancer 1997;75: 324–32.
[13] Mizushina Y, Watanabe I, Ohta K, Takemura M, Sahara H, Takanashi N, Gasa S, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Studies on inhibitors of mammalian DNA polymerase α and β: sulfolipids from a pteridophyte, *Athyrum niponicum*. Biochem Pharmacol 1998;55:537–41.
[14] Ohta K, Miziushina Y, Hirata N, Takemura M, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Sulfoquinovosyldiacylglycerol, KM043, a new potent inhibitor of eukaryotic DNA polymerases and HIV-reverse transcriptase type 1 from a marine red alga, *Gigartina tenella*. Chem Pharm Bull 1998;46:684–6.
[15] Ohta K, Mizushina Y, Hirata N, Takemura M, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Action of a new mammalian DNA polymerase inhibitor, sulfoquinovosyldiacylglycerol. Biol Pharm Bull 1999;22:111–6.
[16] Murakami C, Yamazaki T, Hanashima S, Takahashi S, Takemura M, Yoshida S, Ohta K, Yoshida H, Sugawara F, Sakaguchi K, Mizushina Y. A novel DNA polymerase inhibitor and a potent apoptosis inducer: 2-mono-*O*-(α-D-sulfoquinovosyl)-glyceride with stearic acid. Biochim Biophys Acta 2003;1645:72–80.
[17] Sahara H, Hanashima S, Yamazaki T, Takahashi S, Sugawara F, Ohtani S, Ishikawa M, Mizushina Y, Ohta K, Shimozawa K, Gasa S, Jimbow K, Sakaguchi K, Sato N, Takahashi N. Anti-tumor effect of chemically synthesized sulfolipids based on sea urchin's natural sulfonoquinovosylmonoacylglycerols. Jpn J Cancer Res 2002;93:85–92.
[18] Murakami C, Takemura M, Yoshida H, Sugawara F, Sakaguchi K, Mizushina Y. Analysis of cell cycle regulation by 1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride (SQMG), an inhibitor of eukaryotic DNA polymerases. Biochem Pharmacol 2003;65:259–67.
[19] Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science 1989;246:629–34.
[20] Clarke DJ, Gimenez-Abian JF. Checkpoints controlling mitosis. Bioassays 2000;22:351–63.
[21] Walworth NC. Cell-cycle checkpoint kinases: checking in on the cell cycle. Curr Opin Cell Biol 2000;12:687–704.
[22] Hekmat-Nejad M, You Z, Yee M, Newport JW, Cimprich KA. Xenopus ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint. Curr Biol 2000;10: 1565–73.
[23] Zeng Y, Forbes KC, Wu Z, Moreno S, Piwnica-Worms H, Enoch T. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature 1998;395:507–10.
[24] Zhao H, Watkins JL, Piwnica-Worms H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci USA 2002;99: 14795–800.
[25] Draetta G, Eckstein J. Cdc25 protein phosphatases in cell proliferation. Biochim Biophys Acta 1997;1332:M53–63.
[26] Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature 2000;408:433.
[27] Mizuno T, Ito N, Yokoi M, Kobayashi A, Tamai K, Miyazawa H, Hanaoka F. The second-large subunit of the mouse DNA polymerase α-primase complex facilitates both production and nuclear translocation of the catalytic subunit of DNA polymerase α. Mol Cell Biol 1998;18:3552–62.
[28] Sever-Chroneos Z, Angus SP, Fribourf AF, Wan H, Todeorov I, Knudesen KE, Knudsen ES. Retinoblastoma tumor suppressor protein signals through inhibition of cyclin-dependent kinase 2 activity to disrupt PCNA function in S phase. Mol Cell Biol 2001;21:4032–45.
[29] Uchiyama M, Griffiths D, Arai K, Masai H. Essential role of Sna/Cdc45 in loading of DNA polymerase α onto minichromosome maintenance proteins in fission yeast. J Biol Chem 2001;276: 26189–96.
[30] Woo RA, Poon RY. Cyclin-dependent kinases and S phase control in mammalian cells. Cell Cycle 2003;2:316–24.
[31] Pines J, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell 1989;58:833–46.
[32] Nagata A, Igarashi M, Jinnno S, Suto K, Okayama H. An additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells. New Biol 1991;3:959–68.
[33] Sadhu K, Reed SI, Richardson H, Russell P. Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc Natl Acad Sci USA 1990;87:5139–43.
[34] Galaktionov K, Beach D. Specific activation of cdc25 tyrosine phosphatase by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell 1991;67:1181–94.
[35] Jinno S, Suto K, Nagata A, Igarashi M, Kanaoka Y, Nojima H, Okayama H. Cdc25A is a novel phosphatase is functioning early in the cell cycle. EMBO J 1994;13:1549–56.
[36] Hoffmann I, Draetta G, Karsenti E. Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at G1/S transition. EMBO J 1994;13:4302–10.
[37] Miller JB, Blevitt J, Gerace L, Sadhu K, Featherstone C, Russell P. P55CDC25 is a nuclear protein required for the initiation of mitosis in human cells. Proc Natl Acad Sci USA 1991;88:10500–4.
[38] Seki T, Yamashita K, Nishitani H, Takagi T, Russell P, Nishimoto T. Chromosome condensation caused by loss of RCC1 function requires the cdc25C protein that is located in the cytoplasm. Mol Cell Biol 1992;3:1373–88.
[39] Gabrielli BG, De Souza CP, Tonks ID, Clark JM, Hayward NK, Ellem KA. Cytoplasmic accumulation of cdc25B phosphatase in mitosis triggers centrosomal microtubule. J Cell Sci 1996;109:1081–93.
[40] Baldin V, Cans C, Knibiehler M, Ducommun B. Phosphorylation of human CDC25B phosphatase by CDK1-cyclin A triggers its proteasome-dependent degradation. J Biol Chem 1997;272:32731–4.
[41] Lammer C, Wagerer S, Saffrich R, Mertens D, Ansorge W, Hoffmann I. The cdc25B phosphatase is essential for the G2/M transition in human cells. J Cell Sci 1998;111:2445–53.
[42] Mailand N, Falck J, Lukas C, Syljuasen RG, Welcker M, Bartek J, Lukas J. Rapid destruction of human Cdc25A in response to DNA damage. Science 2000;288:1425–9.
[43] Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 2001;410:842–7.
[44] Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science 1996;274:1664–72.
[45] Boddy M, Furnari B, Mondesert O, Russell P. Replication checkpoint enforced by kinases Cds1 and Chk1. Science 1998;280:909–12.

[46] Weinert T. DNA damage checkpoints update: getting molecular. Curr Opin Genet Dev 1998;8:185–93.
[47] Mailand N, Falk J, Lukas C, Syljuåsen RG, Welcker M, Bartek J, Lukas J. Rapid destruction of human Cdc25A in response to DNA damage. Science 2000;288:1425–9.
[48] Donzelli M, Squatrito M, Ganoth D, Hershko A, Pagano M, Draetta GF. Dual mode of degradation of Cdc25A phosphatase. EMBO J 2002;21:4875–84.
[49] Sampath D, Shi Z, Plunkett W. Inhibition of cyclin-dependent kinase 2 by the Chk1-Cdc25A pathway during the S phase checkpoint activated by fludarabine: dysregulation by 7-hydroxystaurosporine. Mol Pharm 2002;62:680–8.
[50] Feijoo C, Hall-Jackson C, Wu R, Jenkins D, Leitch J, Gillbert DM, Smythe C. Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. J Cell Biol 2001;154:913–23.
[51] Santocanale C, Diffely JF. A Mec-1 and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature 1998;395: 615–8.
[52] Kim SM, Huberman JA. Regulation of replication timing in fission yeast. EMBO J 2001;20:6115–26.
[53] Lindsay HD, Griffiths DJF, Edwards R, Murray JM, Christensen PU, Walworth N, Carr AM. S-phase specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *S. pombe*. Genes Dev 1989;12:382–95.
[54] Tan S, Wang TSF. Analysis of fission yeast primase defines the checkpoint responses to aberrant S phase initiation. Mol Cell Biol 2000;20:7853–86.
[55] Aparicio OM, Weinstein DM, Bell SP. Components and dynamics of MCM proteins and Cdc45p during S phase. Cell 1997;91:59–69.
[56] Feng W, D'urso G. *Schizosaccharomyces pombe* cells lacking the amino-terminal catalytic domains of DNA polymerase epsilon are viable but require the DNA damage checkpoint control. Mol Cell Biol 2001;21:4495–504.
[57] Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998;281: 1677–9.
[58] Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives S, Reiss Y, Shiloh Y, Ziv Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 1998;281:1674–7.
[59] Cortez D, Wang Y, Qin J, Elledge SJ. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science 1999;286:1162–6.
[60] Tibbetts RS, Cortez D, Brumbaugh KM, Scully R, Livingston D, Elledge SJ, Abraham RT. Functional interaction between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev 2000;14:2989–3002.
[61] Yazdi PT, Wang Y, Zho S, Patel N, Lee EY, Quin J. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. Genes Dev 2002;16:571–82.
[62] Kim ST, Xu B, Kastan MB. Involvement of the cohesion protein, Smc1, in Atm-dependent and independent responses to DNA damages. Genes Dev 2002;16:560–70.