INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 21: 281-290, 2008

SPANDIDOS PUBLICATIONS

Inhibitory action of polyunsaturated fatty acids on Cdt1-geminin interaction

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Received October 29, 2007; Accepted December 19, 2007

Abstract. A human replication initiation protein, Cdt1, is a central player in the cell cycle regulation of DNA replication, and geminin down-regulates Cdt1 function by direct binding. It has been demonstrated that Cdt1 hyperfunction resulting from Cdt1-geminin imbalance, for example, by geminin silencing with small interfering RNA, induces DNA re-replication and eventual cell death in some cancer-derived cell lines. We established a high throughput screening system based on a modified enzyme-linked immunosorbent assay to identify compounds that interfere with human Cdt1-geminin binding. Using this system, we screened inhibitors from natural compounds, and found that a fatty acid, linoleic acid (C18:2), from a basidiomycete, inhibited Cdt1-geminin interaction *in vitro*. Of the commercially purchased linear-chain fatty acids tested, the inhibitory effect of oleic acid (C18:1) was the strongest, with 50% inhibition observed at concentrations of 9.6 μM. Since *trans*-configuration, the ester form, and the addition of the hydroxyl group of oleic acid had no influence on C18:1 fatty acid derivatives, both parts of a carboxylic acid and an alkyl chain containing *cis*-type double bonds of fatty acid might be essential for inhibition. Surface plasmon resonance analysis demonstrated that oleic acid was able to bind selectively to Cdt1, but did not interact with geminin. Using a three-dimensional computer modeling analysis, oleic acid was conjectured to interact with the geminin interaction interface on Cdt1, and the carboxyl group of oleic acid was assumed to form hydrogen bonds with the residue of Arg342 of Cdt1. These results suggested that, at least *in vitro*, oleic acid-containing cell membranes of the lipid bilayer inhibit Cdt1-geminin complex formation by binding to Cdt1 and thereby liberating Cdt1 from inhibition by geminin.

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Abbreviations: pre-RC, pre-replication complexes; MCM, mini-chromosome maintenance; ORC, origin recognition complex; CDC6, cell division cycle 6; Cdk, cyclin-dependent kinase; CoQ_{10}, coenzyme Q_{10}; HRP, horseradish peroxidase; GST, glutathione S-transferase; His, six histidine; siRNA, small interfering RNA; ELISA, enzyme-linked immunosorbent assay; RT, room temperature; PBS, phosphate-buffered saline; *o*-PD, *o*-phenylene diamine; DMSO, dimethyl sulfoxide; NP-40, Nonidet P-40; BSA, bovine serum albumin

Key words: fatty acid, oleic acid C18:1, Cdt1, geminin, Cdt1-geminin complex, binding inhibitor, docking simulation, interaction interface

Introduction

In eukaryotic cells, the periodic assembly and disassembly of essential pre-replication complexes (pre-RCs) at replication origins ensure one and only one chromosomal DNA replication (1-3). The pre-RC assembly reaction involves the loading of a presumptive replicative helicase, mini-chromosome maintenance (MCM) 2-7 complexes, onto chromatin by the origin recognition complex (ORC) and two essential factors, cell division cycle 6 (CDC6) and Cdt1 (4,5), which only occur during the low cyclin-dependent kinase (Cdk) period from late mitosis through G1 phase (1-3). At the onset of S phase, Cdk activates the MCM complexes to initiate replication and simultaneously prohibits the re-establishment of pre-RC by suppressing MCM loaders (1-3). One of the suppression mechanisms is the phosphorylation of CDC6, leading to degradation of yeast (6) or nuclear export in mammalian cells (7-10). In human cells, ORC1 and Cdt1 are degraded through phosphorylation by Cdks and subsequent ubiquitination by SCF^{Skp2} ubiquitin ligase (11-14). In addition, there are two other mechanisms that regulate Cdt1; geminin binding (15-18) and replication-coupled, proliferating cell nuclear antigen-dependent proteolysis mediated by the Cullin4-based ubiquitin ligase (19-24).

Such strict regulations of Cdt1 activity in human cells indicate that it is a central player in the regulation of DNA

replication. Indeed, it has been demonstrated that Cdt1 hyperfunction resulting from Cdt1-geminin imbalance, for example, by Cdt1 overexpression or geminin silencing with small interfering RNA (siRNA), induces DNA re-replication and eventual cell death in some cancer-derived cell lines (25-27). On the other hand, in non-transformed cultured cells, Cdt1 overexpression appears not to induce overt re-replication and subsequent growth arrest, although slight chromosomal damage still occurs (28). In addition, many cancer cells constitutively overexpress Cdt1 (28-30); therefore, tumor cells could be selectively eliminated by artificially up-regulating Cdt1 function.

To seek small molecule compounds that inhibit Cdt1-geminin interactions, we previously established a multi-well plate-based, high throughput screening system with recombinant Cdt1 and geminin proteins (31). In principle, compounds that were identified through this screening were classified into at least three categories: (I) compounds that bind to geminin and suppress geminin function as Cdt1 inhibitors (geminin antagonists); (II) compounds that bind to Cdt1 and inhibit Cdt1 function in DNA replication (Cdt1 antagonists); and (III) compounds that bind to Cdt1 but do not inhibit Cdt1 function in DNA replication (indirect geminin antagonists). As discussed above, compounds categorized in (I) or (III), namely geminin antagonists, could become leading compounds for anti-cancer drugs as well as for research reagents to study the Cdt1-geminin system in detail. Recently, new functions of geminin, independent of Cdt1 binding and related to transcriptional regulation, have been successively uncovered (32-35). Therefore, geminin antagonists may also be useful as powerful tools for analyzing such new geminin functions. The compounds categorized in (II), Cdt1 inhibitors, may also be important, for example, in the field of DNA replication research. Using this screening system, we identified coenzyme Q_{10} (CoQ_{10}) as an *in vitro* inhibitor of Cdt1-geminin interaction (31). Further analyses suggested that CoQ_{10} inhibits the formation of the Cdt1-geminin complex by binding to Cdt1 thereby liberating Cdt1 from inhibition by geminin. In addition, CoQ_{10} inhibits the growth of certain human cancer cells, although only at high concentrations (31).

In the present study, we extended the screening of inhibitors to include other natural compounds of microbial fermentation products, and found a potent inhibitor of Cdt1-geminin interaction *in vitro*, a well-known fatty acid, linoleic acid (C18:2) from a basidiomycete (*Ganoderma lucidum*). Subsequently, we investigated the effects of many commercially available fatty acids on the inhibitory activity of Cdt1-geminin binding. We found that several fatty acids, particularly polyunsaturated fatty acids (PUFA) with both long linear-chain and *cis*-configuration, bind or interact with Cdt1 and suppress Cdt1-geminin complex formation. We investigated the inhibitory action of fatty acids on the interaction between Cdt1 and geminin. Furthermore, this study of fatty acids, especially oleic acid (C18:1), may help to clarify the structure and function of Cdt1.

Materials and methods

Materials. Rabbit anti-geminin polyclonal antibody (FL-209) and horseradish peroxidase (HRP)-conjugated forms of mouse anti-rabbit IgG (secondary antibody) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other reagents were of analytical grade and purchased from Nacalai Tesque, Ltd. (Kyoto, Japan).

Fatty acids. The fatty acids were named using the nomenclature described by Weete (36). In the following symbols; (A:B2 ΔC1-C2), A refers to the number of carbon atoms, B2 refers to the number of double bonds, and C1-C2 represents the position of each double bond from the carboxyl end of the molecule. For example, stearic acid, a straight fatty acid that contains 18 carbon atoms and no double bonds, is designated 18:0. The following fatty acids were purchased from Nu-Chek-Prep Inc. (MN, USA): C18-fatty acids such as octadecanoic acid (stearic acid, 18:0), *cis*-9-octadecenoic acid (oleic acid, 18:1 Δ9*cis*), *cis*-9,12-octadecadienoic acid (linoleic acid, 18:2 Δ9-12*cis*), *cis*-9,12,15-octadecatrienoic acid (α-linolenic acid, 18:3 Δ9-12-15*cis*), *cis*-6,9,12,15-octadecatetraenoic acid (parinaric acid, 18:4 Δ6-9-12-15*cis*), *trans*-9-octadecenoic acid (elaidic acid, 18:1 Δ9*trans*), *cis*-9-octadecenoic acid methyl ester (methyl oleate, 18:1 Δ9*cis* methyl) and 12-hydroxy-*cis*-9-octadecenoic acid (18:1 Δ9*cis* 12OH), and other fatty acids such as *cis*-11-dodecenoic acid (12:1 Δ11*cis*), *cis*-9-tetradecenoic acid (myristoleic acid, 14:1 Δ9*cis*), *cis*-10-pentadecenoic acid (15:1 Δ10*cis*), *cis*-9-hexadecenoic acid (palmitoleic acid, 16:1 Δ9*cis*), *cis*-10-nonadecenoic acid (19:1 Δ10*cis*), *cis*-11-eicosenoic acid (gadoleic acid, 20:1 Δ11*cis*), *cis*-13-docosenoic acid (erucic acid, 22:1, Δ13*cis*) and *cis*-15-tetracosenoic acid (nervonic acid, 24:1 Δ15*cis*). To avoid oxidation, the acids were dissolved in *n*-hexane, and stored under nitrogen.

Production of recombinant proteins. Glutathione S-transferase (GST)-fused human Cdt1 (GST-Cdt1) and GST were bacterially produced and purified as described previously (13). Six histidine (His)-tagged human geminin (His-geminin) was synthesized using an *in vitro* transcription-translation system and purified as described previously (13).

Cdt1-geminin binding assay by modified ELISA. The scheme of the assay used to detect binding between human Cdt1 and geminin by enzyme-linked immunosorbent assay (ELISA) and to screen its inhibitors is illustrated in Fig. 1A. Fifty microliters of 20 μg/ml solution of purified GST-Cdt1 in 50 mM carbonate buffer (pH 9.3) was coated on each well of a 96-well plastic microtiterplate (BD Biosciences, San Jose, CA, USA). After incubation for 2 h at room temperature (RT), the plates were blocked with 1% skim milk in PBS (phosphate-buffered saline) for 1 h at RT, and then washed three times in PBS-0.05% Tween-20. Fifty microliters of 0.25 μg/ml purified His-geminin in PBS was added to each well, incubated for 1 h at RT, and then washed twice in PBS. Fifty microliters of rabbit anti-geminin antibody diluted 1:2,000 in PBS-1% skim milk were added to each well, incubated at RT for 1 h, and then washed three times in PBS-0.05% Tween-20. Fifty microliters of mouse anti-rabbit IgG-HRP conjugated (diluted 1:1,000) in PBS-1% skim milk was added to each well and incubated at RT for 1 h. The plates were then washed two times with PBS-0.05% Tween-20 and three times with PBS. The color reaction was developed with

phenylene diamine (*o*-PD) in 50 mM citric acid/Na_2PO_4 buffer containing 0.02% H_2O_2. The reaction was stopped by the addition of 2.5 M H_2SO_4 and the absorbance was read by a microplate reader (Vmax-K, Japan Molecular Devices, Tokyo, Japan) at a test wavelength of 490 nm and a reference wavelength of 630 nm.

Various concentrations of fatty acids in PBS-10% dimethyl sulfoxide (DMSO) were added to the above assay reaction, as shown in Fig. 1A. In particular, 'Cdt1 and fatty acid' or 'fatty acid and geminin' were preincubated at 37˚C for 10 min (i.e., [3] or [4], respectively in Fig. 1A).

Surface plasmon resonance analysis. GST-Cdt1 or His-geminin and fatty acid binding analyses were performed using a Biosensor Biacore instrument (Biacore® 3000) (Biacore, Sweden). CM5 research grade sensor chips (Biacore) were used. All buffers were filtered before use. Purified GST-Cdt1 (95 kDa) or His-geminin (35 kDa) (63.3 or 23.3 μg/ml), respectively, 30 μl each (i.e., 0.2 nmol each) in coupling buffer (10 μM sodium acetate, pH 4.7) was injected over a CM5 sensor chip at 20 μl/min to capture the protein to carboxymethyl dextran matrix of the chip by NHS/EDC coupling reaction (60 μl of mix) as described (37). Unreacted *N*-hydroxysuccinimide ester groups were inactivated using 1 M ethanolamine-HCl (pH 8.0). This reaction immobilized ~5,000-10,000 response units (RU) of these proteins. Binding analysis of a fatty acid, oleic acid, was performed in running buffer including the compound [5 mM potassium phosphate buffer (pH 7.0) and 10% DMSO] at a flow rate of 20 μl/min at 25˚C. Kinetic parameters were determined using BIA evaluation 3.1 software.

Docking simulations between mouse Cdt1 and mouse geminin or oleic acid. Mouse truncated Cdt1 and geminin [Protein Data Bank (PDB) accession code: 1WLQ] were refined by molecular dynamic simulations using Insight II/Discover (Accelrys Inc., San Diego, CA, USA), and all calculations were conducted on SGI workstations, running under the IRIX 6.5 operating system. The binding site of oleic acid on these proteins was determined using the software Insight II/Binding Site Analysis (Accelrys Inc.), and molecular docking of the compound and proteins was performed using a flexible docking procedure in the affinity program within Insight II modeling software (Accelrys Inc.). The calculations used a CVFF force-field in the discovery program and a Monte Carlo strategy in the affinity program (38). Each energy-minimized final docking position of oleic acid was evaluated using the interactive score function in the Ludi module. The Ludi score includes contribution of the loss of translational and rotational entropy of the fragment, number and quality of hydrogen bonds, and contributions from ionic and lipophilic interactions to binding energy.

Results

Effect of fatty acids on the binding between Cdt1 and geminin. For screening *in vitro* inhibitors of human Cdt1-geminin interaction, we used a previously established method (31). As shown in Fig. 1B and C, the positive controls without inhibitors (but including 10% DMSO as a vehicle) were yellow (OD, ~0.845 of absorbance at 490 nm). Negative controls, including GST instead of GST-Cdt1 or excluding either GST-Cdt1 or His-geminin from the reaction, were not colored (OD, ~0.035 of absorbance at 490 nm). Thus, this modified ELISA system was able to specifically detect Cdt1-geminin interaction. The inhibitory activity by commercially purchased linear-chain fatty acids was then investigated using this modified ELISA. Representative data for one of the fatty acids are shown in Fig. 1B and C. C12:1 fatty acid, *cis*-11-dodecenoic acid (12:1 Δ11*cis*), and a methyl ester of C18:1 fatty acid, *cis*-9-octadecenoic acid (oleic acid, 18:1 Δ9*cis*), did not influence binding between Cdt1 and geminin at all. On the other hand, C18:1 fatty acid, *cis*-9-octadecenoic acid (oleic acid, 18:1 Δ9*cis*), significantly inhibited binding. Therefore, the inhibitory effect of various fatty acids was investigated to elucidate the relationship between the structure of linear-chain fatty acids and inhibitory activity.

Inhibitory effect of linear-chain fatty acids on the interaction with Cdt1 and geminin. Since oleic acid (18:1 Δ9*cis*) inhibited the binding between Cdt1 and geminin, commercially purchased C18 fatty acids were investigated for the interaction. As shown in Fig. 2A, 10 and 100 μM of fatty acids were tested. Unsaturated C18 fatty acids of the *cis*-type inhibited the binding, but both the *trans*-configuration of C18:1 fatty acid (i.e., elaidic acid) and C18:0 saturated fatty acid (i.e., stearic acid) did not, suggesting that the *cis*-configuration of double bonds must be important for inhibition. The chemically modified carboxyl group resulting in a methyl ester of C18:1 fatty acid (methyl oleate, 18:1 Δ9*cis* methyl) did not inhibit binding, showing the importance of the free carboxyl group for inhibition. 12-Hydroxy-*cis*-9-octadecenoic acid (18:1 Δ9*cis* 12OH) does not have a chemically modified carboxyl group, but it failed to inhibit the interaction between Cdt1 and geminin. Therefore, a long alkyl chain, such as the hydrophobic region in fatty acids, may be required for inhibition. Of the C18 unsaturated fatty acids, oleic acid (C18:1) showed the strongest inhibition, and the C18 unsaturated fatty acids ranked, in order of their effect, as follows: C18:1>C18:2>C18:3>C18:4. These results suggested that mono-unsaturated fatty acids, which have one double bond, are the best inhibitors of *cis*-type fatty acids. Next we investigated the carbon-chain length of unsaturated fatty acids containing one double bond of *cis*-configuration of the strongest inhibitor of Cdt1-geminin interaction. As shown in Fig. 2B, C18:1 fatty acid (oleic acid) was the strongest inhibitor of the linear-chain C12:1 to C24:1 fatty acids, and the order of their inhibitory effects was as follows: C18:1>C19:1>C20:1>C22:1>C16:1>C24:1>15:1>C14:1>C12:1. Therefore, we concentrated on oleic acid, which was the strongest inhibitor in the later part of this study.

Inhibition by oleic acid of the complex formation between Cdt1 and geminin. Since oleic acid was found to be the most potent inhibitor of Cdt1-geminin interaction among linear-chain fatty acids, we investigated the dose-response curves of this inhibition by oleic acid under various incubation conditions (Fig. 3). In modified ELISA, inhibition was dose-dependent under incubation conditions [1], [3] and [4]

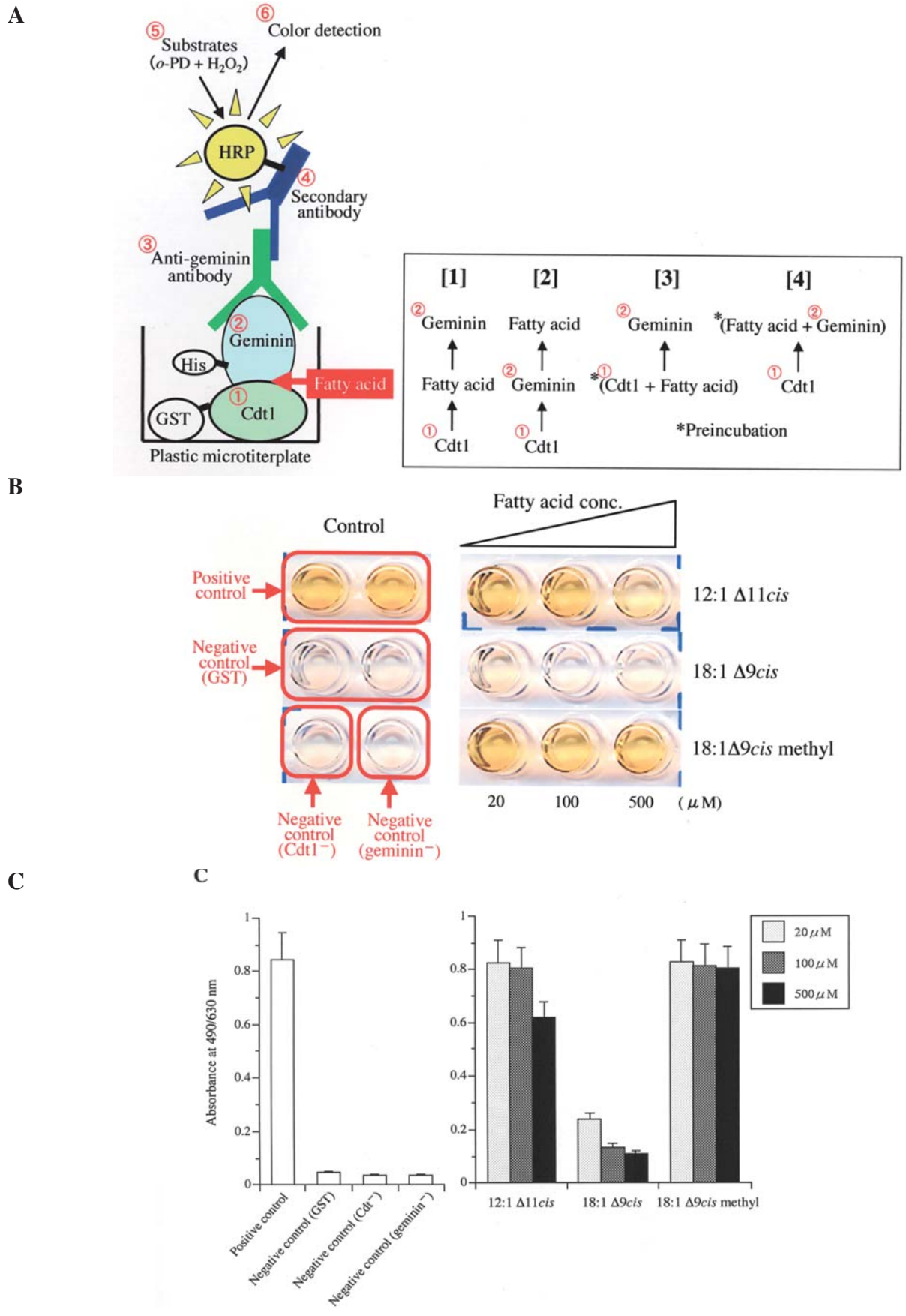

Figure 1. Inhibition of binding between human Cdt1 and geminin by fatty acids. (A) The scheme of the Cdt1-geminin binding assay by modified ELISA. (1) GST-Cdt1, (2) His-geminin, (3) rabbit anti-geminin polyclonal antibody, and (4) horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (secondary antibody). (5) and (6) indicate the color reaction by HRP. [1]-[4] indicate the order of the addition of Cdt1, geminin and fatty acids. Particularly in [3] and [4], 'Cdt1 and fatty acid' or 'fatty acid and geminin' were preincubated, respectively. (B and C) Data of Cdt1-geminin binding inhibition by fatty acids. These results were obtained with the assay method described in [3] of (A). In the 'Positive control', only the vehicle was added. In the 'Negative control (GST)', GST instead of GST-Cdt1 was used. In the 'Negative control (Cdt1⁻)' and 'Negative control (geminin⁻)', Cdt1 and geminin, respectively, were not added. The concentrations of each fatty acid are also shown. (B) represents the raw data of modified ELISA and (C) shows the absorbance data.

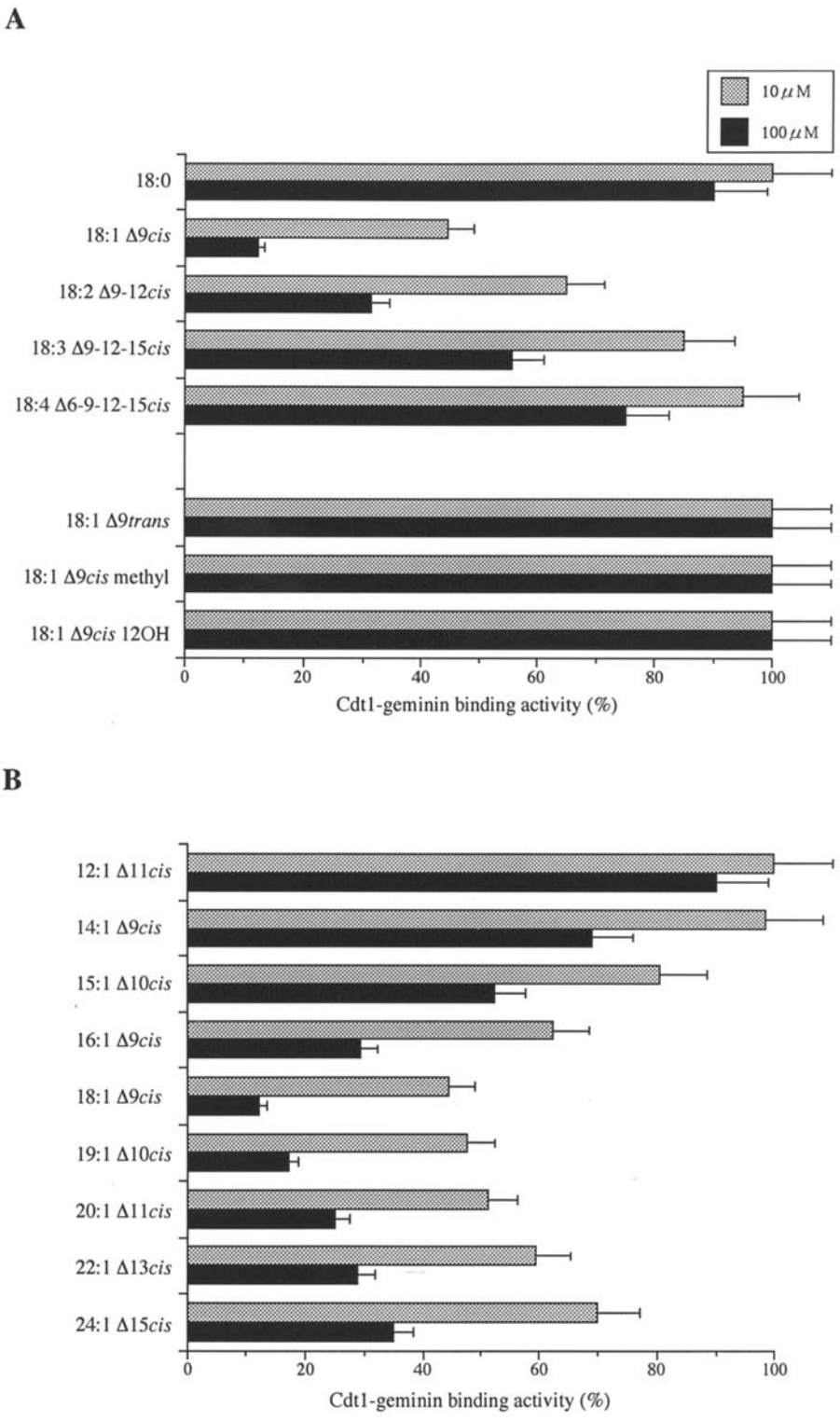

Figure 2. Inhibitory effect of linear-chain fatty acids on human Cdt1-geminin interaction. (A) C18 fatty acids and their derivatives. (B) C12 to C24 mono-unsaturated fatty acids of *cis*-configuration. The value in the absence of fatty acids was taken as 100%. Data are shown as the mean ± SEM of three independent experiments.

(Fig. 1A) with 50% inhibition observed at concentrations of 22.3, 9.6 and 114 μM, respectively. The inhibitory effect of oleic acid under incubation condition [3] was the strongest, and was >2- and 10-fold stronger than under [1] and [4], respectively. On the other hand, oleic acid did not influence binding under the incubation condition [2]. Since Cdt1 and oleic acid were directly mixed and then geminin was added under conditions [1] and [3], oleic acid may have bound or interacted with Cdt1 rather than geminin, thereby inhibiting the formation of the Cdt1 and geminin complex.

Analysis of binding between oleic acid and Cdt1 or geminin. To confirm the kinetic parameters and biochemical experiments precisely, the parameters for binding oleic acid were determined using purified GST-Cdt1 and His-geminin immobilized on a sensor chip in a Biacore. Both proteins

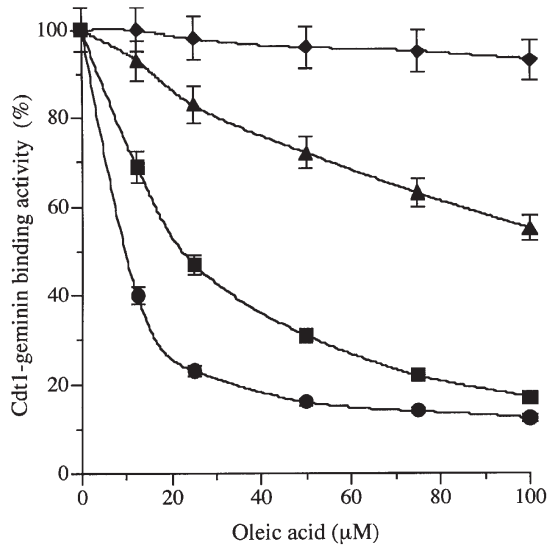

Figure 3. Dose-response curves of oleic acid (0-100 μM) for the inhibition of binding between Cdt1 and geminin. The assay conditions used were [1] (square), [2] (diamond), [3] (circle), and [4] (triangle) as in Fig. 1A, respectively. Inhibitory activity was measured as described in the text. The value in the absence of oleic acid was taken as 100%. Data are shown as the mean ± SEM of three independent experiments.

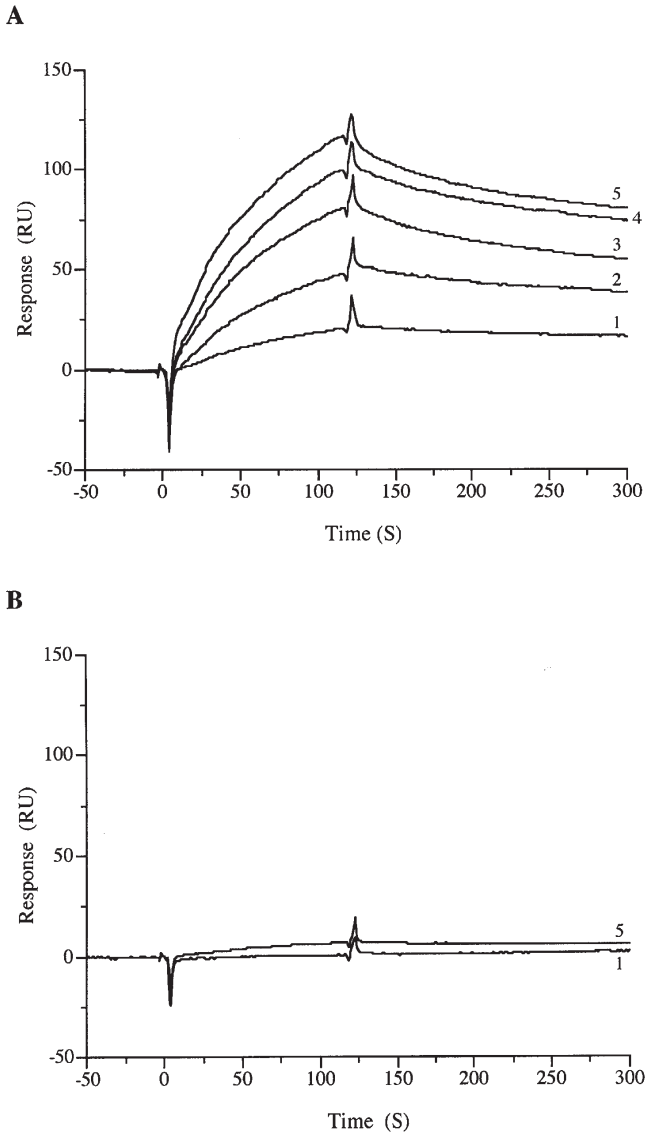

Figure 4. Biacore analysis of the binding of oleic acid to immobilized GST-Cdt1 (95 kDa) (A) or His-geminin (35 kDa) (B). Binding to oleic acid was detected by surface plasmon resonance signal (Biacore, Materials and methods) and is indicated in response units. Five different concentrations of oleic acid (curve 1, 5 μM; curve 2, 10 μM; curve 3, 15 μM; curve 4, 20 μM; curve 5, 25 μM) were injected over the proteins for 120 sec at 20 μl/min and dissociated for 130 sec at 20 μl/min. The background resulting from the injection of running buffer alone was subtracted from the data before plotting.

(0.2 nmol each) were conjugated to the CM5 sensor chip, and then four different concentrations of oleic acid (5, 10, 15, 20 and 25 μM) were added to the conjugated proteins. Oleic acid was able to bind to GST-Cdt1 dose-dependently, and was slightly dissociated from the protein (Fig. 4A). The binding of the molecular ratio between oleic acid and the protein was considered to be 1:1 as indicated by the data. The dissociation constant (K_D) of oleic acid binding to the protein was determined to be 0.823 μM. Biacore analysis demonstrated that GST alone was not bound to 25 μM of oleic acid (data not shown), suggesting that GST had no influence on binding. On the other hand, 25 μM of the compound was unable to bind to His-geminin (Fig. 4B). These results showed that oleic acid directly interacts with Cdt1, and the binding molecular ratio of the compound and the protein is 1:1.

Effect of the reaction condition on Cdt1-geminin binding inhibition. To determine the effects of a non-ionic detergent on the binding of oleic acid to purified GST-Cdt1, Nonidet P-40 (NP-40) was added to the reaction mixture at a concentration of 1.0%. The modified ELISA in this section was performed under condition [3] (Fig. 1A), which involved preincubation of Cdt1 plus oleic acid. In the absence of oleic acid, binding between Cdt1 and geminin was not affected by the addition of NP-40, and we designated the value in these cases as 100%. The inhibitory effect of oleic acid at 100 and 10 μM was moderately and completely reversed by the addition of 1.0% NP-40 to the reaction mixture (Table I). These results suggested that oleic acid was able to interact with the hydrophobic region of the Cdt1 protein. We also tested whether an excess amount of a protein, bovine serum albumin (BSA) (100 μg/ml), could prevent the inhibitory effects of oleic acid. If oleic acid binds to Cdt1 by non-specific adhesion, the addition of the protein would be expected to reduce the inhibition. The fact that BSA did not influence the inhibitory effects of oleic acid (Table I), suggests that the compound selectively binds to a specific site on the Cdt1 protein.

Docking simulation between mouse Cdt1 and mouse geminin or oleic acid. To date, the three-dimensional structure of both Cdt1 and geminin from humans has not been determined by X-ray crystal or NMR analysis, but the cubic structures of the truncated Cdt1-geminin complex from mice are available (PDB accession code: 1WLQ) (Fig. 5A) (18). The proteins used were truncated Cdt1 (tCdt1, residues 172-368; full-length mouse Cdt1 is 1-557) and truncated geminin (tGeminin, residues 79-157; full-length mouse geminin is 1-206). The sequences of Cdt1 and geminin from humans and mice were retrieved from the data bank of the National Center for

effects of bovine serum albumin (BSA) or Nonidet P-40) on the inhibition of binding between human Cdt1 and geminin by oleic acid.

Compounds added to the reaction mixture	Binding activity (%)
Without oleic acid	
None (control)	100±6.8
+ 100 μg/ml BSA	100±7.3
+ 1.0% NP-40	100±7.5
10 μM oleic acid	
10 μM oleic acid	44.4±3.6
10 μM oleic acid + 100 μg/ml BSA	46.0±4.0
10 μM oleic acid + 1.0% NP-40	94.5±8.5
100 μM oleic acid	
100 μM oleic acid	12.3±1.1
100 μM oleic acid + 100 μg/ml BSA	11.8±1.2
100 μM oleic acid + 1.0% NP-40	76.9±7.0

The incubation condition was as [3] in Fig. 1A. BSA (100 μg/ml) or 1.0% NP-40 was added to the reaction mixture of Cdt1 and oleic acid. The binding activity of Cdt1 and geminin in the absence of oleic acid was taken as 100%. Data are expressed as the mean ± SD; n=4.

Biotechnology Information (NCBI). The amino acid homology of Cdt1 and geminin between humans and mice was investigated using the method of composition-based statistics, and these scores are calculated below:

Cdt1: identities = 404/563 (71%), positives = 442/563 (78%), gaps = 28/563 (4%)

Geminin: identities = 162/208 (77%), positives = 181/208 (87%), gaps = 3/208 (1%)

The identity values of both Cdt1 and geminin showed very high scores, implying that the three-dimensional structure of the Cdt1-geminin complex from mice is essentially the same as that of humans. Thus, for oleic acid, which was the strongest binding inhibitor of Cdt1 and geminin of the linear-chain fatty acids, docking simulation was performed using the mouse truncated Cdt1-geminin (tCdt1-tGeminin) complex instead of the human proteins.

As shown in Fig. 5A, the N-terminal region of a coiled-coil homodimer of tGeminin (residues 113-119) interacts with both the N-terminal and C-terminal parts of tCdt1 (i.e., residues 181-192 and 322-346, respectively) (18). The secondary interface (residues 322-346) relies on steric complementarity between the tGeminin homodimer and the hydrophobic face of the C-terminal H6-helix and L2-loop of tCdt1 (18).

First, the three-dimensional binding structure between tCdt1 and oleic acid was studied. The oleic acid-binding site of tCdt1 was refined using software Insight II/Binding Site Analysis, and the molecular ratio of oleic acid and tCdt1 was simulated as 1:1 from the data of surface plasmon resonance analysis (Fig. 4A). As a result, the oleic acid-binding region in the pocket of the protein could be mapped to one face of tCdt1 consisting of hydrophobic rather than hydrophilic amino acid residues (the blue and white surface of tCdt1 in Fig. 5B). This oleic acid-binding pocket was contained in the interaction interface of tGeminin (i.e., C-terminal residues 322-346 of tCdt1).

Table II. Binding energy of oleic acid-interacting amino acids residues of Cdt1.

Oleic acid interacting amino acid	Energy (kcal/mol)		
	Coulomb	van der Waals	Total
Pro331	0.288	-1.791	-1.503
Met333	0.288	-2.826	-2.538
Leu340	-0.923	-1.986	-2.909
Arg342	-108.163	2.631	-105.532
His344	-1.857	-4.417	-6.274
Arg346	-27.003	-2.477	-29.480

All amino acids in mouse truncated Cdt1 (residues 172-368), which interacted with oleic acid, are indicated. The binding energy was calculated by the flexible docking procedure in the affinity program within the Insight II modeling software (Accelrys Inc., San Diego, CA, USA).

The alkyl chain of oleic acid was significantly shifted following the binding to tCdt1 by the flexible docking procedure in the affinity program within Insight II modeling software, and was able to interact with the hydrophobic H6-helix and L2-loop of tCdt1 (Fig. 5B and C). The oleic acid-interacted amino acid residues of tCdt1 and their binding energies are indicated in Table II. The binding energy of van der Waals of the residues of Pro331, Met333, Leu340 and His344 was stronger than that of coulomb, suggesting that these residues could interact with the alkyl chain of oleic acid by hydrophobic force (Fig. 5D). In particular, because the van der Waals force of His344 was the strongest of all the amino acid residues of tCdt1 (-4.417 kcal/mol), this residue must strongly interact with oleic acid by a hydrophobic bond. The tCdt1 surface of the alkyl chain of oleic acid binding consists of the C-terminal H6-helix and L2-loop, which makes contact with the tGeminin homodimer (i.e., residues 322-346 of tCdt1) (18), and the six amino acid residues of tCdt1, indicated in Table II, directly bind to both tGeminin and oleic acid (Fig. 5D). The inhibitory effect of oleic acid on Cdt1-geminin binding activity was reversed by the addition of 1.0% NP-40 to the reaction mixture (Table I), suggesting that the interaction between oleic acid and Cdt1 must be a hydrophobic effect. On the other hand, since the coulomb binding energy of the residues of Arg342 and Arg346, which are hydrophilic amino acids, was stronger than the van der Waals force, these residues may bind to the carboxyl group of oleic acid by hydrophilic force (Fig. 5D). In particular, the coulomb force of the Arg342 residue was the strongest of all of the amino acids of tCdt1 (-108.163 kcal/mol), and this residue could bind to the compound by a hydrogen bond (the dotted line in Fig. 5D). The total binding energy between the Arg342 residue and the oleic acid molecule was the

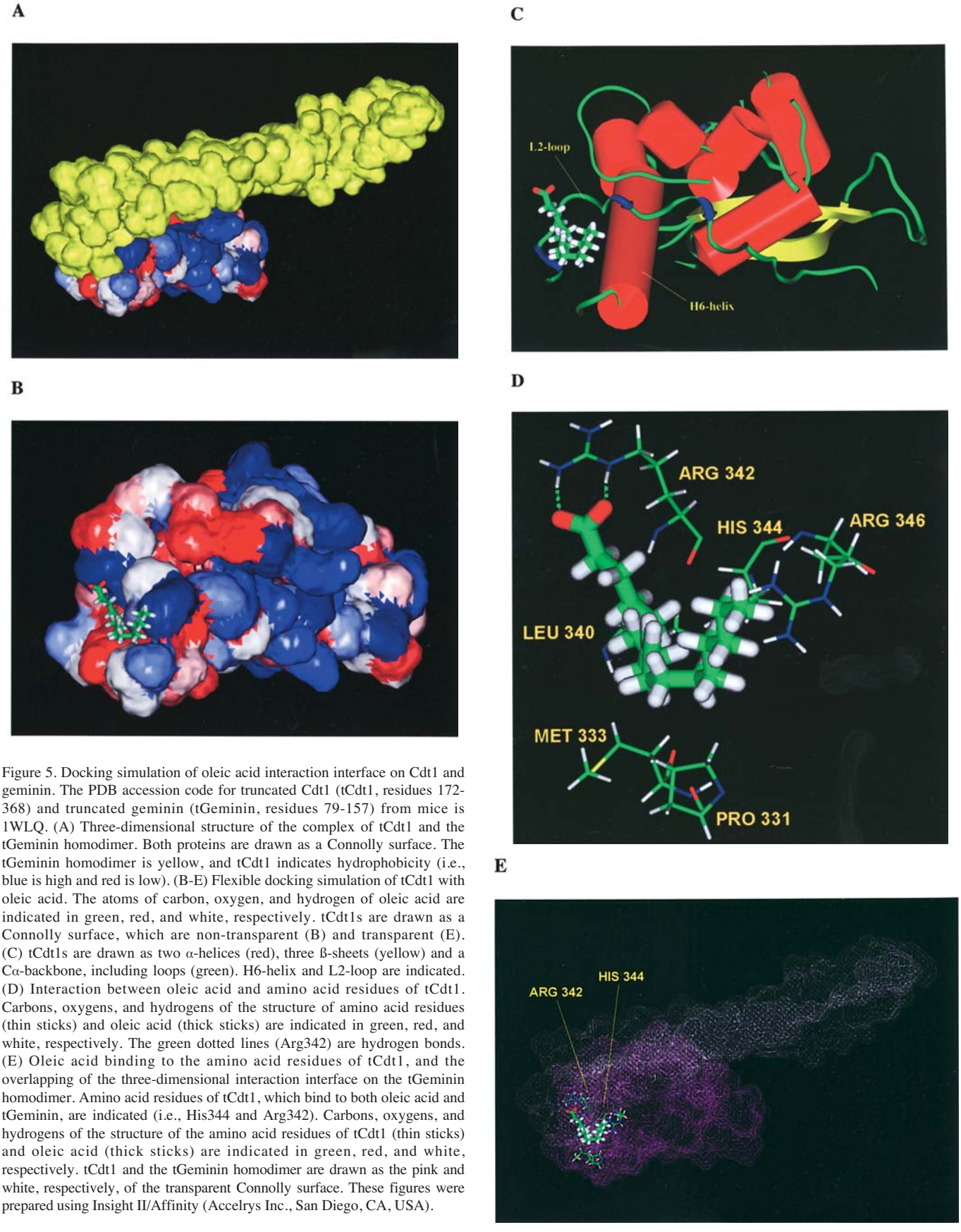

Figure 5. Docking simulation of oleic acid interaction interface on Cdt1 and geminin. The PDB accession code for truncated Cdt1 (tCdt1, residues 172-368) and truncated geminin (tGeminin, residues 79-157) from mice is 1WLQ. (A) Three-dimensional structure of the complex of tCdt1 and the tGeminin homodimer. Both proteins are drawn as a Connolly surface. The tGeminin homodimer is yellow, and tCdt1 indicates hydrophobicity (i.e., blue is high and red is low). (B-E) Flexible docking simulation of tCdt1 with oleic acid. The atoms of carbon, oxygen, and hydrogen of oleic acid are indicated in green, red, and white, respectively. tCdt1s are drawn as a Connolly surface, which are non-transparent (B) and transparent (E). (C) tCdt1s are drawn as two α-helices (red), three ß-sheets (yellow) and a Cα-backbone, including loops (green). H6-helix and L2-loop are indicated. (D) Interaction between oleic acid and amino acid residues of tCdt1. Carbons, oxygens, and hydrogens of the structure of amino acid residues (thin sticks) and oleic acid (thick sticks) are indicated in green, red, and white, respectively. The green dotted lines (Arg342) are hydrogen bonds. (E) Oleic acid binding to the amino acid residues of tCdt1, and the overlapping of the three-dimensional interaction interface on the tGeminin homodimer. Amino acid residues of tCdt1, which bind to both oleic acid and tGeminin, are indicated (i.e., His344 and Arg342). Carbons, oxygens, and hydrogens of the structure of the amino acid residues of tCdt1 (thin sticks) and oleic acid (thick sticks) are indicated in green, red, and white, respectively. tCdt1 and the tGeminin homodimer are drawn as the pink and white, respectively, of the transparent Connolly surface. These figures were prepared using Insight II/Affinity (Accelrys Inc., San Diego, CA, USA).

strongest (-105.532 kcal/mol), and this amino acid residue in the L2-loop of tCdt1 strongly and directly binds to the tGeminin homodimer (18). Therefore, Arg342 must be important for the formation of the Cdt1-geminin complex. As shown in Fig. 5E, both the carboxyl moiety and the alkyl chain of oleic acid may compete with geminin to bind to the residues of Arg342 and His344 of Cdt1, respectively, and prevents contact between Cdt1 and geminin.

In this study, we found that C18:1 fatty acid, oleic acid, inhibits Cdt1-geminin interaction *in vitro* at relatively lower concentrations (IC_{50} value of ~10 μM) (Fig. 3). Surface plasmon resonance analyses clearly showed that oleic acid binds to Cdt1 and not to geminin (Fig. 4); therefore, it would be classified as either a Cdt1 antagonist or indirect geminin antagonist. We suggest that oleic acid acts as an indirect geminin antagonist as follows. Computer simulation indicates that oleic acid may bind to the Cdt1 surface that forms the binding interface with geminin and thereby inhibits Cdt1-geminin interaction (Fig. 5B-E). Geminin binds to the N-terminal and middle portions (i.e., residues 181-192 and 322-346) of Cdt1 via the N-terminus. However, this binding itself is insufficient for Cdt1 inhibition, which may be carried out by the C-terminus of geminin by interfering with MCM-Cdt1 interaction at the Cdt1 C-terminus (18). Indeed, a truncated geminin mutant lacking the N-terminus can bind to Cdt1 but does not inhibit Cdt1 activity to load MCM (18). We therefore speculate that oleic acid may not inhibit Cdt1 activity and is able to act as a geminin antagonist. In line with this notion, oleic acid did not inhibit MCM-Cdt1 interaction *in vitro* (data not shown). These properties of oleic acid for the inhibition of Cdt1-geminin interaction appear similar to those of CoQ_{10}, as we reported previously (31); however, computer simulation analyses indicated that their binding pockets on Cdt1 are different. These data may help to further understand the structure and function of Cdt1.

We found that linear-chain unsaturated fatty acids of *cis*-type, especially oleic acid, strongly inhibited Cdt1-geminin interaction *in vitro*. The chemical frames of fatty acids containing double bonds could, moreover, be used for screening new anti-cancer chemotherapy agents. They can be three-dimensionally pursued by using data on the structural heterogeneity of the fatty acid-binding pockets of the target protein, since fatty acids, including conjugated fatty acids, can be chemically synthesized in great variety. Therefore, the computer-simulated drug design of compounds, especially the binding inhibitors of Cdt1 and geminin, could be of great interest, and may in theory be a promising approach to developing new agents for anti-cancer chemotherapy.

Acknowledgements

This work was supported, in part, by a grant-in-aid for Kobe-Gakuin University Joint Research (A), 'Academic Frontier' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology of Japan), 2006-2010, (to H.Y. and Y.M.), and a grant (17080013) from MEXT (to M.F.). Y.M. acknowledges the grants-in-aid from 19680031 for Young Scientists (A) MEXT (Japan), and the Nakashima Foundation (Japan).

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