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Peptide binding to Geminin and inhibitory for DNA replication☆

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Abstract

Geminin binds to Cdt1 to ensure that DNA replication occurs only once during the cell cycle. To identify the peptide that binds to Geminin and thereby modifies the latter's ability to alter the DNA replication activity in human cancer cells, we screened a phage display library of random peptides in successive cycles of phage library panning and found one peptide sequence that bound to the 31–111 amino acid residues of Geminin. Delivery of this peptide sequence into the nucleus of HCT116 human colon cancer cells resulted in the suppression of BrdU incorporation. These results provide new insights into the function of Geminin and further validate Geminin as a potential therapeutic target in tumors.

Keywords: Phage display; Geminin; DNA replication; Peptide

Strict regulation of DNA replication is essential for the accurate propagation of genetic information, as aberrant chromosome replication may give rise to ploidy or mitotic defects, which can result in tumorigenesis or cell death [1]. DNA replication is controlled by the stepwise assembly of a pre-replicative complex (pre-RC) and the replication apparatus [2–4]. Geminin is a newly discovered component of the pre-RC and plays a role in preventing incorporation of the minichromosome maintenance protein (MCM) complex, an essential DNA helicase, into the pre-RC via binding and inhibiting Cdt1 function [5–7]. Thus, an important regulatory mechanism other than that mediated by the cyclin-CDKs to prevent re-replication in higher eukaryotes is provided by Geminin [5]. Geminin contains a destruction box sequence near its amino terminus, that is necessary for anaphase-promoting complex-dependent ubiquitination and mitotic degradation [5].

A non-degradable form of Geminin, in which the destruction box was mutated, in human cancer cells activated the DNA checkpoint machinery and arrested cellular proliferation, suggesting that stabilization of Geminin inhibits cancer cell proliferation [8–10]. Accumulating evidence suggests that Geminin is overexpressed in certain tumors, suggesting that disturbance of the Geminin/Cdt1 balance could potentially promote tumorigenesis [9,11].

On the basis of such information, it may be possible to design low-molecular-weight chemicals that would bind to Geminin to suppress the aberrant cellular proliferation in tumor cells. Identifying such peptides and their target domains could lead to predictions as to the design of potential low-molecular-weight inhibitors of aberrant DNA replication. Here, we report that a certain peptide sequence isolated by screening a random peptide phage library that bound to Geminin causes suppression of BrdU incorporation in human cancer cells. This study suggests that the chemical peptidomimetics of this peptide might form the basis for the development of drugs that could be used to prevent tumor progression.

Materials and methods

Production of proteins. A plasmid encoding the full-length human Geminin was kindly provided by Dr. Anindya Dutta (University of Virginia) [10]. Fragments encoding the full-length of Geminin (FL; 1–212 aa), lacking the N-terminal destruction box (Δ1–30), lacking the

☆ *Abbreviations:* pre-RC, pre-replicative complex; GST, glutathione-*S*-transferase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

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destruction box and the potential neuralization domain (Δ1–111), lacking the coiled-coil domain and the rest of the C-terminal region (Δ112–212), or lacking the C-terminal region (Δ146–212) were amplified by PCR, digested with *Eco*RI and *Xho*I, and cloned into the bacterial expression vector, pGEX-6P-1 (Amersham). The identities of all of these constructs were confirmed by DNA sequencing on an Applied Biosystems 377A automatic DNA sequencer. The primer sequences are available on request. The mouse Cdt1 expression vector, pET-FLAG-mCdt1-His_6, was kindly provided by Dr. Fumio Hanaoka (Osaka University), and expression and purification of the recombinant protein in *Escherichia coli* was performed according to a previously published method [12].

Freshly transformed BL21 Star (DE3) *E. coli* cells (Invitrogen) were grown in LB medium containing 0.1 g/L ampicillin and the *lac* promoter was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.5. Expression was allowed to continue for 3.5 h at 26 °C. Cells were harvested by centrifugation at 4000*g* for 15 min at 4 °C. Cell extracts were prepared with the BugBuster HT protein extraction reagent (Novagen) plus a protease inhibitor cocktail (Calbiochem) under constant agitation for 15 min at room temperature and centrifuged at 15000*g*, followed by filtration. The supernatants were applied to a GSTrap FF column (Amersham) according to the manufacturer's instructions. The purified proteins were electrophoresed on a NuPAGE 4–12% Bis–Tris gel with the Mes running buffer (Invitrogen) and visualized by GelCode Blue staining (Pierce).

GST pull-down assay. Fifty nanograms of glutathione-*S*-transferase (GST)–Geminin protein and Flag-mCdt1-His protein was mixed with glutathione–Sepharose (Amersham) for 4 h at 4 °C in 100 μl binding buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 0.1% Nonidet P-40. The Geminin-binding peptide was also added at an increasing concentration. After washing with binding buffer, GST-tagged proteins were eluted with 30 μl Laemmli sample buffer. Equal volumes of the eluted protein were electrophoresed on a NuPAGE 4–12% Bis–Tris gel with the Mes running buffer (Invitrogen) and transferred to a Hybond-PVDF membrane (Amersham). The membrane was first blocked in PBS containing 0.1% Tween 20 and 5% non-fat dried milk, and then incubated with anti-GST polyclonal (Amersham) and anti-Flag monoclonal (Sigma) antibodies. Enhanced chemiluminescence reagents were used to detect the signals according to the manufacturer's protocol (ECL Plus, Amersham).

Phage selection and ELISA. A random 12-mer peptide phage library (Peptide Door, Japan) was used for the selections. Phage was selected by panning on immunotubes (Nunc) coated with recombinant Geminin at 10 μg/ml. Selections were performed essentially as described previously [13]. Positive clones were identified by a screening phage produced from single colonies for specific binding to GST–Geminin in ELISA. An ELISA plate was coated overnight at 4 °C with GST–Geminin at 20 μg/ml in 100 mM $NaHCO_3$, pH 8.0. After blocking the plate with PBS plus 2% skimmed milk powder (blocking buffer) for 1 h at RT, the plate was washed five times with PBS–0.05% Tween 20 (PBST), and 16 μl of phage supernatant together with 64 μl of blocking buffer was added to the plate pre-inoculated with 120 μl PBS for 1 h at RT. The plate was then washed five times with PBST, and phage binding was detected using horseradish peroxidase-conjugated anti-M13 monoclonal antibody (Amersham) with 2,2′-azino-di-(3 ethylbenzthiazoline sulfonic acid) (ABTS, Sigma) and H_2O_2 as a substrate. To exclude phage clones that bind to the GST, GST–Geminin, and GST were immobilized to the plates individually. The absorbance value (*A*) was determined by subtraction of the raw *A* of the GST plates from that of the GST–Geminin plates. The wavelength for the absorbance detection was set at 410 nm.

Peptide synthesis. We arranged for the synthetic coupling of fluorescent-labeled peptide (WIATWQDDGYMY) to the SV40 nuclear-localizing signal (PKKKRKV) at Multiple Peptide Systems

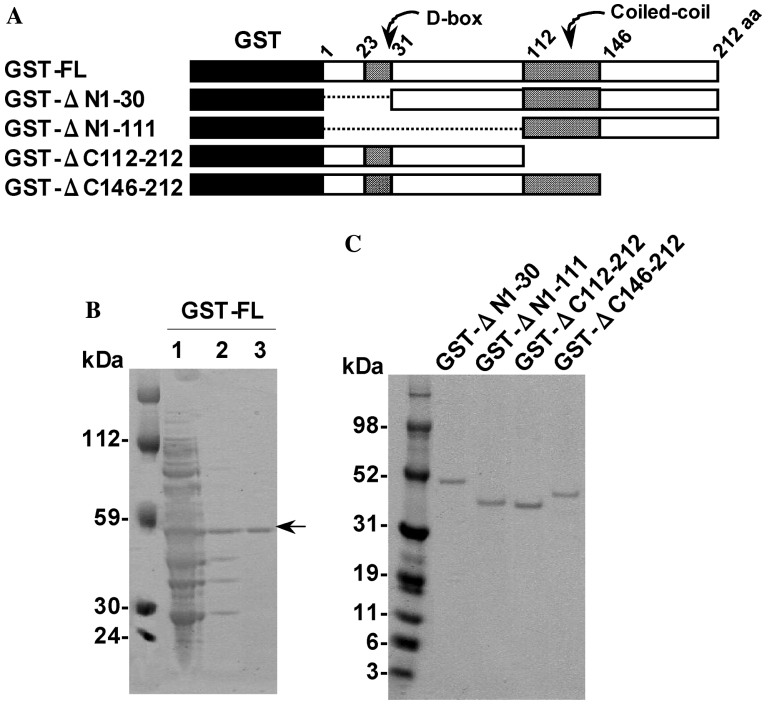

Fig. 1. Expression and purification of bacterially expressed Geminin. (A) Recombinant Geminin constructs. The GST tag is depicted by filled boxes. Numbers indicate amino acid residues. Deletion regions are shown by dotted lines. The destruction box (D-box) and coiled-coil region are depicted by shaded boxes. (B) Purification of recombinant Geminin from *E. coli*. Extracts from *E. coli* (lane 1) were fractionated sequentially over glutathione–Sepharose (lanes 2 and 3), and bound proteins were eluted with an elution buffer containing 100 mM glutathione. Proteins were analyzed by NuPAGE 4–12% gradient gels and stained with Coomassie brilliant blue. The arrow indicates the position of GST-full-length Geminin (FL). (C) Purified Geminin deletion mutants. A series of truncated Geminin proteins was prepared from *E. coli*, as described in (B).

(San Diego). Control peptide, RSQRGRAPRGRGRKC, was synthesized at Peptide Laboratories (Osaka) and labeled with the ProtOn Fluorescein-labeling kit (Vector Laboratories). All peptides were synthesized by solid-phase synthesis, using Fmoc chemistry. The peptides were purified by gel filtration and the products were found to be more than 85% pure, according to the results of HPLC analysis.

Cell culture, transfections, peptide visualization, and BrdU incorporation. HCT116 cells (ATCC) were cultured in McCoy's 5A medium (Invitrogen) containing 10% (v/v) fetal bovine serum (FBS) (Invitrogen) and antibodies (penicillin and streptomycin), at 37 °C under 5% CO_2. The cells were transfected with the Pro-Ject protein transfection reagent kit (Pierce) according to the manufacturer's instructions. Briefly, 10 μM of fluorescein-labeled peptide and Pro-Ject protein transfection reagent mixed together with 250 μl of serum-free culture medium per 8-well Lab-Tek II chamber slide (Nalge Nunc) was used for each transfection. At 4 h after transfection, one volume of 20% serum-containing culture medium supplemented with 40 μM BrdU (Roche) was added directly to the well, and the cells were incubated for 20 h at 37 °C. The cells were washed three times with PBS, fixed with ethanol for 30 min at 4 °C, and washed three times again with PBS. The cells were sequentially blocked with 1% bovine serum albumin–0.01% Tween 20 in PBS for 5 min. They were then rinsed in PBS–0.05% Tween 20, incubated for 1 h with anti-BrdU monoclonal antibody (Roche), washed, and incubated for 45 min with anti-mouse IgG conjugated to Cy3 (Zymed Laboratories). These samples were examined under a fluorescence microscope IX70 (Olympus).

Results and discussion

Isolation and characterization of the peptide binding to Geminin

As illustrated in Fig. 1A, Geminin can be divided into three functional domains: a conserved coiled-coil motif at the carboxyl terminus, a neuralization domain at the amino terminus [14], and a destruction box for ubiquitin-mediated degradation at its amino terminus [5]. We used the full-length Geminin protein as a target for the panning of the phage display library of 12-mer random peptides. Geminin was expressed as a GST-tagged protein in bacteria and purified to near homogeneity (Fig. 1B). After several rounds of biopanning, we isolated a single unique sequence, WIATWQDDGYMY. This sequence did not show any homology to known proteins or motifs in the databases. We next used ELISA to test which region of Geminin may be responsible for the affinity of the protein to the isolated phage clone. Synthetic mutants lacking the destruction box (D-box, ΔN1–30), the destruction box and the potential neuralization domain (ΔN1–111), the coiled-coil domain and the rest of C-terminal region (ΔC112–212), and the C-terminal region (ΔC146–212) were expressed and purified (Fig. 1C). Among the tested Geminin deletion proteins, GST-ΔN1–111 as well as GST gave low absorbance values in the ELISA assay, indicating that the amino acid residues 31–111 are responsible for the peptide binding (Fig. 2A).

The region containing the amino acid residues 31–79 has been reported as a potential neuralization domain

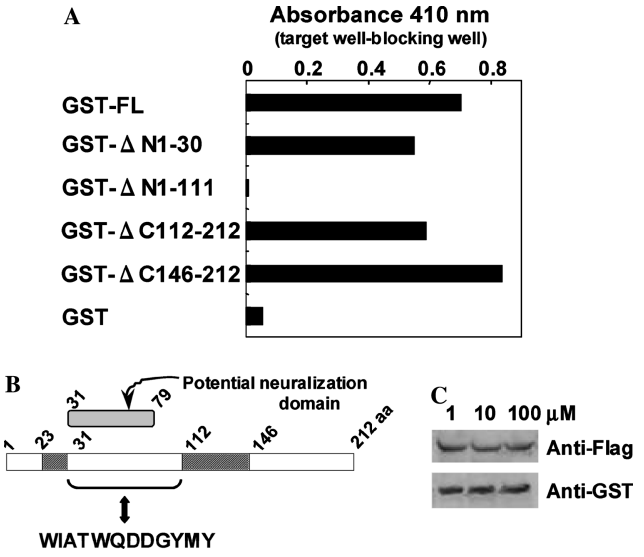

Fig. 2. Characterization of the Geminin-binding peptide. (A) Epitope mapping using different Geminin fragments. The absorbance value (*A*) was determined by subtraction of the raw *A* of the GST plates from that of the GST–Geminin plates. The wavelength for absorbance detection was set at 410 nm. (B) Schematic representation of the domain organization of Geminin. Interaction domains required for binding of the peptide to Geminin are shown. The potential neutralization domain is depicted by a gray box. Numbers indicate amino acid residues. The destruction box (D-box) and coiled-coil region are also shown by shaded boxes. The Geminin-binding peptide, WIATWQDDGYMY, is depicted near its binding region with Geminin. (C) Pull-down analysis of Flag-tagged Cdt1 with GST-tagged Geminin. Purified GST-tagged Geminin fusion protein (50 ng) was mixed with purified Flag-tagged Cdt1 (50 ng) and increasing concentrations of the Geminin-binding peptide and incubated with glutathione–Sepharose to precipitate complexes containing Cdt1 proteins. The complexes were washed and visualized by Western blotting using anti-Flag and anti-GST antibodies.

using the *Xenopus* embryo [14] (Fig. 2B), and also in *Drosophila*, overexpression of Geminin in the embryo inhibited DNA replication and induced ectopic neural differentiation [15]. Therefore, the Geminin–peptide interaction may modify neural differentiation. Within the region responsible for the Geminin–peptide binding, several potential phosphorylation sites have been shown [16]. These include serine 49, a potential phosphorylation site for protein kinase C or casein kinase I, serine 45 and threonine 59 for the glycogen synthase kinase 3β, and serine 63 and serine 85 for the casein kinase I. Future studies of the effects of the peptide on Geminin should reveal whether or not it acts as an inhibitor of the specific phosphorylation sites. Next, we examined whether or not increasing the amount of the peptide (~100 μM) interfered with the Geminin–Cdt1 association. For this purpose, we produced the Flag-tagged Cdt1 protein in bacteria, and the Geminin–Cdt1 association was examined by the GST pull-down assay. The Geminin-binding peptide could not prevent the interaction between Geminin and its binding protein Cdt1 (Fig. 2C).

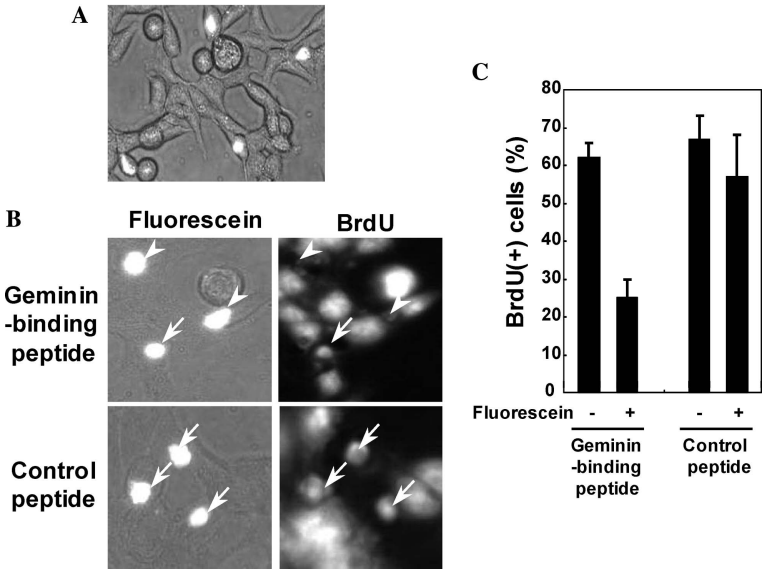

Fig. 3. Geminin-binding peptide inhibits DNA synthesis in HCT116 human colon cancer cells. (A) HCT116 cells were transfected with fluorescein-labeled Geminin-binding peptide linked to the SV40 nuclear-localizing sequence peptide. Accumulation of the peptide into the nucleus was observed. Fluorescence microscopy was performed on live cells (100×). (B) HCT116 cells were transfected with fluorescein-labeled Geminin-binding or control peptide. Fluorescence microscopy was performed on the fixed cells (200×). The same fields were examined for BrdU incorporation. White arrows indicate the fluorescein(+) cells that were positive for BrdU incorporation; arrowheads indicate the fluorescein(+) cells that were negative for BrdU incorporation. (C) The proportion of BrdU(+)/fluorescein(+) and BrdU(+)/fluorescein(−) cells was determined by analyzing at least 50 fluorescein(+) or (−) cells for each transfection in three fields, in two independent experiments, and the SD values were determined.

Inhibitory effect of the peptide on BrdU incorporation

The peptides were conjugated to an SV40-derived nuclear-localizing signal peptide known to localize to the nucleus [17] and then conjugated with fluorescein for monitoring the intracellular localization of the peptides. After incubation of the cells with peptide–cationic lipid complexes for 24 h, approximately 10% of the cells were found to be positive for fluorescein-mediated fluorescence in the nucleus of the HCT116 human diploid colon cancer cells (Fig. 3A).

Because Geminin plays an important role in the assembly of pre-RC and initiation of DNA replication, we investigated the effect of the Geminin-binding peptide on the DNA replication activity. By detecting fluorescein fluorescence to mark specific transfected cells, we examined the effects of the peptide transfection on the synthesis of new DNA in HCT116 cells. We found fewer BrdU(+) cells following transfection of the Geminin-binding peptide than following that of the control peptide (Fig. 3B). A quantitative summary of the percentage of BrdU(+)/fluorescein(+) and BrdU(+)/fluorescein(−) cells from independent experiments is shown in Fig. 3C. Geminin-binding peptide inhibited new DNA synthesis in HCT116 cells by approximately 50% as compared with that noted following transfection of the control peptide. Our data are the first to suggest inhibition by a Geminin-binding peptide of the DNA replication activity based on a cell-based assay.

Our aim was to identify the peptide that binds to the Geminin protein to modify the latter's ability to alter the DNA replication activity in human cancer cells. It is interesting to note that in a study using human lymphoma cells, increased levels of Geminin were associated with increased tumor cell proliferation [9]. Therefore, the peptide reported here might have a deleterious effect on Geminin. Although the precise mechanism of the inhibition of DNA replication by the Geminin–peptide binding remains unclear, the isolation of this peptide may form the basis for further studies to delineate in detail the interaction between Geminin and other pre-RC components. Finally, this peptide may also serve as the lead compound for a rational design of low-molecular-weight products useful against tumorigenesis.

Acknowledgments

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