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Developing peptide-based multivalent antagonists of proliferating cell nuclear antigen and a fluorescence-based PCNA binding assay

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is a critical player in cell proliferation. It interacts with a myriad of cellular proteins in genomic DNA replication and cell cycle control. This makes PCNA an attractive target for developing antiproliferative therapeutics. Indeed, the binding of a human tumor suppressor protein, p21, to PCNA contributes to its antiproliferative effect in cells. In this work, we report a fluorescence polarization-based binding assay for determining the affinity between the p21 peptide and human PCNA. To improve the potency of the p21-based PCNA antagonist, we exploited the homotrimeric structure of PCNA and developed multivalent peptide-based PCNA antagonists. The di- and trivalent p21-based antagonists bind to PCNA with low nanomolar dissociation constant. Moreover, we show that the multivalent PCNA antagonists inhibited PCNA-dependent DNA synthesis in a human cell extract with improved avidity when compared with the monovalent p21 peptide. The fluorescence polarization assay holds promise for the discovery of potent small-molecule PCNA inhibitors given its ready adaptability to a high-throughput screening format.

Controlled cell proliferation is crucial for the normal growth of human tissue and organs. Abnormality in cell proliferation usually leads to neoplastic diseases and cancer. Normal cell proliferation requires the orchestrated actions of proteins from various essential cellular pathways, including DNA replication, DNA repair, and cell cycle regulation [1,2]. Numerous studies have pointed to the proliferating cell nuclear antigen (PCNA)[1] as a master regulator of cell growth in humans [3]. PCNA possesses the striking ability of interacting with a myriad of proteins functioning in DNA replication/repair, translesion DNA synthesis, chromatin remodeling, and cell cycle checkpoint regulation [4,5]. The unique properties of PCNA make it an attractive target for controlling eukaryotic cell proliferation. Indeed, the human tumor suppressor protein, p21, exerts inhibitory effects on cell growth by targeting PCNA [6,7]. Several studies have shown that depression of PCNA expression can effectively inhibit cell growth [8,9]. During the past decade, there have been impressive advances in understanding the structural and functional properties of PCNA [3]. The valuable information derived from these studies set the stage for developing potent PCNA antagonists for controlling cell proliferation. The development of effective PCNA antagonists will also contribute to the treatment of cancer and other common diseases such as rheumatoid arthritis [10]. Furthermore, the development of PCNA antagonists may find valuable application in tissue engineering given that regulating and preventing the neoplastic outgrowth of implanted tissue is important for the success of tissue regeneration [11–13].

Proliferating cell nuclear antigen, as suggested by its name, was initially identified as an abundant protein species in proliferating cells. Later, X-ray crystallography revealed that PCNA belongs to a universal protein family called "clamp" protein, which functions as a processivity factor for the replicative DNA polymerase [5,14]. One important function of PCNA is to greatly increase the processivity of DNA polymerase by tethering it to the DNA template. PCNA also orchestrates a number of other essential cellular pathways by recruiting numerous proteins to the replication fork [3]. Many of these proteins bind to PCNA through a PIP (PCNA interacting protein) box located either internally or at the terminus of the protein sequence [4]. The PIP box sequence can be generalized to Qxx(M/L/I)xxF(Y/F) (where x stands for variable amino acid). A previous study suggested that the affinity of PCNA binding protein is influenced by the variable residues of the PIP box as well as the sequence that flanks the PIP box [15].

A previous study demonstrated that suppression of PCNA expression by antisense oligonucleotides selectively inhibits gastric cancer cell proliferation [8]. A similar observation was made for

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[1] *Abbreviations used:* PCNA, proliferating cell nuclear antigen; PIP, PCNA interacting protein; IDC, interdomain connector; PCR, polymerase chain reaction; IPTG, isopropyl β-D-1-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; Ni–NTA, nickel–nitrilotriacetic acid; ESI, electrospray ionization; MS, mass spectrometry; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; CD, circular dichroism; UV, ultraviolet.

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smooth muscle cells in rat carotid artery injury models by suppressing PCNA expression [9]. Therefore, PCNA represents an attractive target for controlling cell proliferation in different cell types. It is known that the human tumor suppressor protein, p21, exerts an inhibitory effect on cell growth by binding to PCNA [6]. p21 is a member of the CDK inhibitor protein family [16]. The N-terminal region of p21 binds to cyclins and inhibits the cyclin-dependent kinases that function at the major transition points of the cell cycle [17,18]. The C-terminal region of p21 binds directly to the interdomain connector (IDC) loop binding site on PCNA. The binding of p21 peptide to PCNA blocks the association of DNA polymerases with PCNA, thereby preventing DNA synthesis. Notably, a 20-amino-acid peptide spanning the C-terminus of p21 was found to be sufficient for interaction with PCNA. Overexpression of this peptide in mammalian cells was able to arrest cell division [19,20]. Subsequent biochemical studies revealed that p21 peptide binds to PCNA tightly, with a dissociation constant of 88 nM [21].

Given the potential of p21 peptide in controlling cell proliferation, several laboratories have developed peptides that bind to PCNA with an improved affinity [15,21,22]. Interestingly, it was found that shortening of the p21 peptide resulted in a severe loss of binding affinity to PCNA. To circumvent this problem, peptides with shorter length were rationally designed based on the consensus sequence of the PIP box. However, only one of the designed peptides demonstrated a slightly higher binding affinity [21]. Another study employed peptides derived from various PCNA binding proteins, including the p66 subunits of human DNA polymerase δ and the human FEN1 endonuclease [15]. However, the affinity constant of the peptides was found to be at least 200-fold lower compared with the p21 peptide [15]. Random peptide libraries were also investigated to select for PCNA binding peptides [23,24]. Although novel peptides were identified, no significant improvement in binding affinity was achieved. The limited success in developing highly potent PCNA binding peptides stresses the need for new design strategies that are different from the strategies employed previously.

Numerous biological processes in nature involve multivalent binding interactions. In many cases, the binding affinity between individual molecules is often weak; however, multivalent interactions function to increase the binding affinity and stabilize complex formation [25,26]. Exploiting multivalency, researchers have developed a number of polyvalent inhibitors targeting various proteins and receptors [27–30]. In most studies, multivalent interactions were of significantly higher affinity compared with their respective monovalent counterpart.

In the current work, we have developed a method to monitor PCNA binding by fluorescence polarization. With this new assay method, we quantified the binding affinity between a fluorescently labeled p21 peptide and human PCNA. Moreover, a fluorescence-based competition assay was developed to quantify the binding affinity between the PCNA antagonists and PCNA. To improve the affinity of the p21-based PCNA antagonist, we exploited the multiple binding sites on the homotrimeric PCNA and generated multivalent antagonists consisting of two or three repeats of the native p21 peptide. Both di- and trivalent p21 peptides bound to PCNA with improved affinity. We also demonstrate that the multivalent p21-based peptides effectively inhibit DNA synthesis in a human cell-free extract. Our results support the notion that PCNA antagonists with improved efficacy can be attained by exploiting the multivalency of the PCNA binding sites.

Materials and methods

Plasmid construction and gene cloning

The human PCNA gene was amplified by polymerase chain reaction (PCR) using human complementary DNA (cDNA) as a template. The amplified PCR product was cloned into the *Escherichia coli* expression vectors pET22b and pET28a using the restriction sites *Nde*I and *Xho*I. The PCNA/pET22b plasmid encodes human PCNA without tag, whereas the PCNA/pET28a plasmid allows the expression of human PCNA with an N-terminal 6×His tag. To generate the expression plasmids for the p21 divalent peptide (KRRQTSMTDFYHSKRRLIFSGGSGGSGGSGGSGGSGGSKRRQTSMTDFYHSKRRLIFS) and the p21 trivalent peptide (KRRQTSMTDFYHSKRRLIFSGGSGGSGGSGGSGGSGGSKRRQTSMTDFYHSKRRLIFSGGSGGSGGSGGSGGSKRRQTSMTDFYHSKRRLIFS), a synthetic gene was constructed by GenScript where repeats of the p21 peptide were linked together by flexible glycine/serine linkers. The amplified gene products were cloned into the *E. coli* expression vector pET28a using the restriction sites *Nde*I and *Xho*I. The di- and trivalent p21/pET28a plasmid allowed the expression of peptides with an N-terminal 6×His tag.

Protein expression and purification

Human PCNA was expressed in Rosetta(DE3) cells (Novagen). Cells were cultured at 37 °C until the OD_{600} reached 0.8. The cell culture was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultured for 15 h at 17 °C following the induction. For PCNA with no tag, the cells were harvested and sonicated in lysis buffer (50 mM Tris [pH 7.5], 50 mM NaCl, 5% glycerol, 2.5 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride [PMSF]). The cell-free extract was loaded onto a DEAE Sepharose column equilibrated with wash buffer (50 mM Tris [pH 7.5], 5% glycerol, and 2.5 mM β-mercaptoethanol) at a flow rate of 2.0 ml/min. The human PCNA was eluted off the column using a salt gradient between 170 and 250 mM NaCl. Protein purity was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining. The concentrated fractions were diluted in the wash buffer to a final NaCl concentration of 50 mM and then loaded onto a HiTrap Q FF anion exchange column (GE Healthcare) at a flow rate of 2.0 ml/min. PCNA was eluted off the column at a NaCl concentration of 340 mM. The PCNA fractions were pooled together and dialyzed against a buffer containing 10 mM Na_2HPO_4 (pH 7.5), 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, 5% glycerol, and 0.5 mM dithiothreitol (DTT). Protein purity was analyzed using SDS–PAGE and Coomassie blue staining.

For purification of the 6×His-tagged PCNA, the cells were resuspended and sonicated in lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF, and 10 mM imidazole). The cell-free extract was incubated with nickel–nitrilotriacetic acid (Ni–NTA) resin (Invitrogen) and washed extensively with lysis buffer. Bound PCNA was eluted with lysis buffer containing 100 mM imidazole. Eluted PCNA fractions were combined and diluted to a final NaCl concentration of 100 mM using a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 5% glycerol, and 1 mM β-mercaptoethanol. Diluted protein solution was loaded onto a HiTrap Q FF anion exchange column (GE Healthcare) at a flow rate of 2.0 ml/min. Bound PCNA was eluted off the column at a NaCl concentration of 350 mM. Pure fractions were combined and buffer exchanged into a buffer containing 10 mM Na_2HPO_4 (pH 7.5), 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, 5% glycerol, and 0.5 mM DTT. Protein purity was analyzed using SDS-PAGE and Coomassie blue staining.

The di- and trivalent p21 peptides were expressed in the Rosetta(DE3) cells (Novagen). Cells were cultured at 37 °C until the OD_{600} reached 0.8. The cells were then induced with 0.4 mM IPTG and were cultured for an additional 8 hours at 37 °C. The cells were harvested and sonicated in lysis buffer [50 mM NaH_2PO_4 (pH 8.0), 8 M urea, 500 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, and 10 mM imidazole]. Cell free extract was bound to Ni-NTA resin