The role of primer recognition proteins in DNA replication: inhibition of cellular proliferation by antisense oligodeoxyribonucleotides

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Summary

Primer recognition proteins (PRP) are cofactors for DNA polymerase α and may have a role in lagging-strand DNA replication. PRP is composed of two subunits, which we have previously identified as the protein-tyrosine kinase substrate annexin II and phosphoglycerate kinase (PGK). In this study, we have examined the physiological involvement of these proteins in DNA synthesis and cell proliferation. When exponentially growing human HeLa cells are exposed to antisense phosphorothioate oligodeoxynucleotides to annexin II, ongoing DNA synthesis is reduced. The extent of reduction with antisense oligodeoxynucleotide to PGK was much less than with the antisense annexin II oligodeoxynucleotide. Reductions in the labeling and mitotic indices of HeLa cell cultures are seen after exposure to antisense oligodeoxynucleotides. Flow cytometric analyses indicate that progression from S phase to G2 phase of the cycle is retarded by exposure of cells to the antisense oligodeoxynucleotides. Corresponding sense oligodeoxynucleotides have no inhibitory effects on these parameters. The new synthesis of annexin II and PGK is specifically reduced in the presence of antisense oligodeoxynucleotides, indicating that the complex of newly synthesized annexin II and PGK may participate in PRP function. These experiments indicate that annexin II and PGK may have a physiological role in DNA synthesis and cell cycle progression, and represent the first physiological role for annexin II monomer in cells.

Key words: oligodeoxyribonucleotides, primer recognition proteins, DNA replication.

Introduction

Primer recognition proteins (PRP) enable DNA polymerase α to utilize efficiently template-primers with low primer to template ratios, a situation found on the lagging-strand DNA template at the replication fork (Pritchard and DePamphilis, 1983). PRP has been purified to homogeneity from HeLa cells (Jindal and Vishwanatha, 1990a) and is composed of two subunits, which have been identified as PGK (Jindal and Vishwanatha, 1990b) and annexin II (also called calpactin I, p36 or lipocortin II) monomer (Jindal et al., 1991). PGK and annexin II are abundant cellular proteins and we find that only a small subset of these two proteins are associated together as the PRP complex (Jindal et al., 1991). To determine the physiological involvement of these proteins in DNA synthesis, we have used multiple approaches. Immunoelectron microscopic analysis has revealed specific association of these two proteins with nuclei in normal and transformed cells (Kumble and Vishwanatha, 1991). We find that nuclear PRP is tightly associated with the nuclear scaffold (Vishwanatha et al., 1992). Immunodepletion of annexin II from Xenopus laevis egg extracts results in loss of replicative ability, which can be restored by addition of purified annexin II (Kumble, S. and Vishwanatha, J. K. in preparation). Enhanced expression of annexin II is seen in pancreatic cancers of Syrian hamsters and the enhanced expression is associated with proliferating areas of the cancer and in metastatic foci (Kumble et al., 1992). In this study we have examined the physiological role of annexin II and PGK in DNA synthesis by utilizing the antisense oligodeoxynucleotide approach, whereby the functions of each of these proteins are measured in the DNA synthesis process.

Antisense oligodeoxynucleotides have been used as modulators of gene expression in a number of model systems, and they have also been used in inhibition of human immunodeficiency virus replication and expression of viral antigens in infected cell cultures (Zamecnik et al., 1986), inhibition of cell proliferation by PCNA/cyclin (Jaskulski et al., 1988), inhibition of human immunodeficiency virus type 1 mediated cytopathic effects (Stevensen and Iversen, 1989) and inhibition of expression of SV40 large T-antigen
that PRP plays a physiological role in DNA synthesis many types (Stein and Cohen, 1989). Our data indicate the phosphorothioate analogs of the oligodeoxyribonucleotides because of the known resistance of their internucleoside linkages to degradation by nucleases of many types (Stein and Cohen, 1989). Our data indicate that PRP plays a physiological role in DNA synthesis and cell proliferation.

Materials and methods

Cells
HeLa cells were maintained at 37°C in an atmosphere of 10% CO2 in Dulbecco's MEM (DMEM) supplemented with 5% defined iron-supplemented calf serum (HyClone Laboratories, Logan, UT).

Preparation of phosphorothioate oligodeoxyribonucleotides
The sense and antisense phosphorothioate oligodeoxyribonucleotides were synthesized essentially as described by Stein et al. (1990). Briefly, oligodeoxyribonucleotide synthesis was performed on a 1 μmol scale by use of an Applied Biosystems model 380B DNA synthesizer and the recommended cycle for hydrogen phosphonate chemistry, which includes activation of the monomer with adamantane carboxyl chloride and capping with isopropyl phosphate that is activated similarly. The support-bound, 5'-dimethoxytrityl (DMT) 20-mer hydrogen phosphonate chemistry, which includes activation and cell proliferation.

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Oligonucleotide toxicity assay
Human HeLa cells were plated at 5 x 10^4 cells/well in 96-well microtiter plates and various concentrations of the sense or antisense oligodeoxyribonucleotides (4 μM-60 μM final concentration) and of the alkylating agent bis-(2-chloromethyl)-amine hydrochloride (1.25-160 μM) were added and incubated for 24 hours at 37°C. The culture medium was removed and the cells were rinsed once with the culture medium followed by the addition of 200 μl of 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) dissolved in culture medium and the plates were incubated for 2 hours. The cells were rinsed with phosphate-buffered saline (PBS) and 200 μl of dimethyl sulfoxide was added and plates incubated at 37°C for 45 minutes. The contents in each well were mixed and the plates were read on a Molecular Devices V-max plate reader at 540 nm.

Measurement of DNA synthesis
HeLa cells were plated at 4 x 10^4 cells/well of a 96-well plate in 2 ml of DMEM with 5% fetal bovine serum and incubated at 37°C for 24 hours, following which the medium was removed and replaced with 1 ml of DMEM without serum, containing various concentrations of the oligodeoxyribonucleotides. After a 30 minute incubation, fetal bovine serum was added to a 10% final concentration. This was done to eliminate any possibility of degradation of oligodeoxyribonucleotides by serum components, despite the known resistance of phosphorothioate oligodeoxyribonucleotides to nucleolytic attack. At various time points, [3H]thymidine (6 μCi/well) was added and further incubated for 30 minutes. DNA synthesis was measured by extracting DNA from cell cultures on an Applied Biosystems (model 340A) DNA extractor.

Measurement of labeling and mitotic indices
HeLa cells were seeded and incubated with oligodeoxyribonucleotides as described above. For measurement of labeling indices, harvested cells were washed once with PBS, pelleted and resuspended in 200 μl of 33% (v/v) glacial acetic acid in methanol. A sample of the fixed cells was spotted on a clean glass slide in triplicate and allowed to air dry for 30 minutes. The slides were soaked in cold 10% trichloroacetic acid for 10 minutes and then rinsed in distilled water. The slides were coated with NTB-2 tracking emulsion (Eastman Kodak) and exposed in the dark for two weeks, following which they were developed and stained with Giemsa. Numbers of cells with autoradiographic grains per 100 total cells were counted and the data are shown as % of cells labeled. For the measurement of mitotic indices, the procedure of Peterson et al. (1979) was followed. Briefly, the harvested cells were resuspended in 2 ml of Hank's balanced salt solution (HBSS) containing 15 μl of colchicine solution (10 mg of colchicine was dissolved in 0.67 ml ethanol and 0.96 ml propylene glycol; this volume was made up to 10 ml with 0.17 M Na2HP04/0.1 M NaH2PO4; before use, 1 ml of the stock colchicine was diluted in 24 ml of HBSS). After incubation at room temperature for 3 hours, 1 ml of colchicine was removed and 4 ml of distilled water was added. The tube was allowed to stand for 10 minutes followed by the addition of 0.7 ml of 33% (v/v) glacial acetic acid in methanol with constant agitation. The cells were pelleted and resuspended in fresh fixative. A sample of the cells was spotted on a clean glass slide, allowed to air dry and stained with Giemsa. The number of cells in mitosis per 100 total cells was then counted.

Flow cytometry
Exponentially growing HeLa cells were exposed to sense and antisense oligodeoxyribonucleotides as described above, for various times. The cells were fixed and stained with Vindelov's reagent (3.4 mM Tris-HCl, pH 7.6, 10 mM NaCl, 0.7 i.u./ml ribonuclease A, 0.1 mM propidium iodide and 0.1% Nonidet P-40). Cell cycle analysis was performed on an Ortho 50H cytofluorograf.

Measurement of new synthesis of annexin II and PGK
HeLa cells were seeded and incubated with oligodeoxyribonucleotides as described above. At various time points, [35S]methionine (0.5 mCi/ml) was added and the cells were incubated further for 2 hours. Cells were dislodged by trypsinization, pelleted and washed with PBS. The cell pellet was lysed with 50 μl of lysis buffer (0.5% Nonidet P40, 0.5% nonanol-N-methylglucamide, 150 mM NaCl, 5 mM EDTA, 2
mM PMSF and 5 mM iodoacetamide in 50 mM Tris-HCl, pH 7.5) for 45 minutes at 4°C. The suspension was clarified by centrifugation and the supernatant was used. A 20 μg sample of protein was electrophoresed through a 4% to 15% gradient SDS-polyacrylamide gel, electrotransferred onto Immobilon-P membrane and immunoblotted with rabbit antisera to human PGK or chicken annexin II according to published procedures (Jindal et al., 1991). The blots were developed with alkaline phosphatase-conjugated goat anti-rabbit IgG. Densitometric analyses of the blots were performed on a BioRad scanning densitometer and the radioactivity in the annexin II and PGK bands was determined on a Betascope 603 (Betagen) radioanalytical imager.

Results

Oligodeoxyribonucleotides to PRP
Oligodeoxyribonucleotides complementary to either the sense or the antisense strand of the annexin II and PGK sequence were constructed and their sequences are given in Fig. 1. The oligodeoxyribonucleotides to PGK start 2 nucleotides preceding the AUG codon and extend to 20 bases in length. The oligodeoxyribonucleotides to annexin II comprise 20 bases beginning from the 2nd base of the AUG codon. The antisense strands are complementary strands of the sense strands. The phosphorothioate oligonucleotides were dissolved in culture medium without serum and used in our experiments.

The toxicity of oligodeoxyribonucleotides to HeLa cells was tested using an enzyme activity-based MTT assay in which a colored formazan product is only produced by reducing enzymes in living metabolically active cells (Cole, 1986). No cell killing was observed with sense and antisense oligodeoxyribonucleotides to either annexin II or PGK at concentrations from 0.4 μM to 60 μM. As a positive control in these experiments, we used an alkylating agent, bis-(2-chloroethyl)amine hydrochloride, at concentrations of 1.25-160 μM. Complete cell killing was observed at concentrations of 80-160 μM. No cell killing was observed at concentrations of 60 μM, the oligodeoxyribonucleotides were non-toxic to cells. These experiments were repeated on the Chang cell line and we observed identical results. In further experiments, we used HeLa cells and a concentration of 30 μM of each oligodeoxyribonucleotide. Phosphorothioate oligodeoxyribonucleotides are stable in cultured cells and tissues from rats (Iversen, P., personal communication).

Effect of antisense oligodeoxyribonucleotides on DNA synthesis
In these experiments, HeLa cells were exposed to 30 μM concentrations of either the sense or antisense oligodeoxyribonucleotide to annexin II or PGK for various intervals. The incorporation of [3H]thymidine (6 μCi/ml) into DNA in 30 minutes of incubation prior to harvesting the cells was monitored. DNA was isolated on a Applied Biosystems (model 340A) DNA extractor. Addition of antisense oligodeoxyribonucleotide to annexin II markedly inhibited the ability of cells to incorporate the label into DNA (Fig. 2). Exposure to antisense PGK oligodeoxyribonucleotide resulted in 20-30% reduction in DNA synthesis (data not shown). A rapid decline in ability to synthesize DNA upon exposure to annexin II antisense oligodeoxyribonucleotide was seen after as little as six hours of incubation and continued throughout the course of our experiments. Cells treated with antisense oligodeoxyribonucleotides slowly recovered from the inhibitory effects especially at the 40 hour time point examined. Two possible explanations for this observation are that the cells are exposed to oligodeoxyribonucleotides only once at time 0 and over the course of the experiment, the concentration of oligo may be reduced gradually; secondly, the availability of oligodeoxyribonucleotides in the nuclei may be reduced by the annexin II antisense oligodeoxyribonucleotide was seen.

Fig. 2. Effect of antisense oligodeoxyribonucleotide to annexin II on incorporation of [3H]thymidine to DNA. Logarithmically growing HeLa cells were incubated with 30 μM of sense (filled symbols) or antisense oligodeoxyribonucleotide (open symbols) to annexin II monomer. At the indicated time points, [3H]thymidine (6 μCi/ml) was added for 30 minutes. DNA was extracted from the cell cultures using a Applied Biosystems model 340A DNA extractor and the radioactivity was determined by scintillation spectrometry. The data are normalized to cts min⁻¹ incorporated per unit of DNA as determined by UV absorbance at 260 nm.

Fig. 1. Oligodeoxyribonucleotide sequences and their relationship to the cDNA sequences of PRP. The cDNA sequence of annexin II monomer (Huang et al., 1986) and PGK (Michelson et al., 1985) are from the literature.
PGK oligodeoxynucleotide may represent a limited role for this protein as a primer recognition protein.

Effect of antisense oligodeoxynucleotides on cell proliferation

The labeling indices of HeLa cell cultures exposed to either the sense or antisense oligodeoxynucleotides are shown in Fig. 3. Cells exposed to antisense oligodeoxynucleotides to annexin II (Fig. 3A) and PGK (Fig. 3B) had markedly decreased labeling indices. The decrease is observed at all the time points used. In cells exposed to corresponding sense oligodeoxynucleotides, the labeling index remained high and at about the same level as that in untreated control cultures. Stimulation of the labeling index in the presence of sense oligodeoxynucleotides has been observed upon exposure of cells to antisense PCNA oligodeoxynucleotides (Jasikulski et al., 1988). The pattern presented in Fig. 3 is reproducible by repeated experimentation. The number of autoradiographic grains per nucleus varied from cell to cell. There were no significant differences between cultures treated with sense or antisense oligodeoxynucleotides. The difference we observed is in the percentage of labeled cells in each treatment. While the extent of the decrease in labeling index with antisense PGK oligodeoxynucleotide is similar to that with antisense annexin II oligodeoxynucleotide (Fig. 3), we did not observe a corresponding decrease in DNA synthesis with antisense PGK oligodeoxynucleotide. The reason for this is unclear. Our earlier studies (Jindal et al., 1991) indicated that for PRP activity in vitro, both PGK and annexin II are necessary. Thus, exposure of cells to antisense oligodeoxynucleotide directed against one of the components may result in reduced formation of the complex and thereby affect labeling indices to a similar extent.

The effect of these oligodeoxynucleotides on cell cycle progression and cell proliferation was measured by counting the percentage of cells in mitosis and representing this as the mitotic index. The data shown in Fig. 4 indicate that the mitotic indices are significantly reduced by the
Antisense oligo inhibition of DNA synthesis

Addition of antisense oligodeoxynucleotides as compared to the corresponding sense oligodeoxynucleotide controls for both annexin II (Fig. 4A) and PGK (Fig. 4B). With the antisense annexin II oligodeoxynucleotide, significant decrease in mitotic index occurred at the 12 hour and 18 hour time points. At the corresponding 12 hour time point, annexin II oligodeoxynucleotide had very little effect, while at the 24 hour time point, this oligodeoxynucleotide had a significant decrease in mitotic index in repeated experiments. The reason for the lack of effect at the earlier time points and the significant decrease at the 24 hour time point is unclear. The mitotic index of cultures treated with sense oligodeoxynucleotides was not significantly different from the corresponding controls. While variations were observed from experiment to experiment in the absolute numbers, the pattern of inhibition was reproducible by various repetitions.

Flow cytometric analysis of cells treated with sense and antisense oligodeoxynucleotides to annexin II (Fig. 5) indicated that the antisense oligodeoxynucleotide treatment of cells results in a slow progression of cells through the S phase. The population of cells in S phase increases (at 24 and 36 hours) and a corresponding decrease in the G2 population is seen. These data are consistent with the reduction in labeling and mitotic indices seen with the antisense oligodeoxynucleotides. No significant differences were observed between sense and antisense oligodeoxynucleotides in the G0-G1 population. The populations of cells in different cell cycle phases upon exposure of cells to sense oligodeoxynucleotides were very similar to that in untreated control.

New synthesis of PGK and annexin II

The effect of oligodeoxynucleotides on the new synthesis of annexin II and PGK was measured after treatment of cells with the oligodeoxynucleotides for 12 or 24 hours. Cell cultures were labeled with [35S]methionine (0.5 mCi/ml) for 2 hours and the labeled cell extract was electrophoresed on SDS-polyacrylamide gels and immunoblotted using specific antibodies. The ratio of [35S]methionine label to the densitometric reading of the corresponding bands represents the fraction of each protein that was synthesized during the labeling period. Fig. 6 illustrates the results of these experiments. There were no marked differences in the extent of labeling or in the types of proteins labeled in the presence of sense or antisense oligodeoxynucleotides (Fig. 6A). Immunoblot analyses using anti-annexin II oligodeoxynucleotides (Fig. 6B) and anti-PGK oligodeoxynucleotides (Fig. 6C) show a specific decline in the levels of the two proteins in the presence of corresponding anti-sense oligodeoxynucleotides. New synthesis of annexin II (Fig. 6D) is reduced in cultures treated with antisense oligodeoxynucleotide as compared to the sense oligodeoxynucleotide at the two time points observed. Similar results were observed in experiments with oligodeoxynucleotides to PGK also (Fig. 6E). The reduction in new annexin II (Fig. 6D) corresponds to the reduction in DNA synthesis (Fig. 2) at the 12 hour and 24 hour time points.

Discussion

Our results indicate that inhibition of new synthesis of annexin II and PGK reduces the ability of cells to undergo DNA synthesis and thus these proteins may have a physiological role in cellular DNA synthesis. Nonsynchronized cells appear to contain abundant and fairly uniform amounts of these two proteins, suggesting that their concentrations do not vary markedly during the cell cycle. The use of antisense oligodeoxynucleotides provides a direct test of the role of these proteins in DNA synthesis and cell proliferation. Antisense oligodeoxynucleotide to annexin II reduces the capability of cells to synthesize DNA and to progress through the cell cycle. Significantly less inhibition is observed with antisense PGK oligodeoxynucleotide, indicating a limited role for this protein in the PRP complex. Ability to inhibit cell proliferation by exposure of cells to antisense oligonucleotides has been shown with c-fos (Holt et al., 1986), c-myc (Heikkila et al., 1987) and PCNA/cyclin (Jaskulski et al., 1988). In the presence of antisense oligodeoxynucleotide to c-myc, human lymphocytes do not enter the S phase of cell cycle after mitogen stimulation (Heikkila et al., 1987) and differentiation is induced in HL-60 cells (Holt et al., 1988). In contrast to the results with PCNA/cyclin (Jaskulski et al., 1988), we did not see a complete cessation of cell proliferation, but a reduction in the ability of cells to synthesize DNA and progress through the cell cycle. At later time points (24-36 hours), the effect of antisense was reduced. Reversible inhibition of growth and restoration of growth after removal of the oligodeoxynucleotide have been observed before (Jaskulski et al., 1988; Heikkila et al., 1987). Sense oligodeoxynucleotide to annexin II was found to stimulate DNA synthesis (Fig. 2). This stimulation is probably due to DNA repair synthesis. By treatment of
Fig. 6. New synthesis of annexin II and PGK after treatment of cells with sense and antisense oligodeoxynucleotides. HeLa cells were exposed to sense or antisense oligodeoxynucleotides to annexin II or PGK for 12 or 24 hours. Prior to harvesting, cells were labeled with [35S]methionine (0.5 mCi/ml) for 2 hours, cell extract was prepared and PRP proteins were analyzed as described under Materials and methods. (A) An autoradiogram of 35S-labeled proteins in the presence of sense (+) or antisense (−) oligodeoxynucleotide to PGK for 12 or 24 hours. (B and C) Corresponding immunoblots for annexin II (B) and PGK (C). New synthesis of annexin II (D) and PGF (E) in the presence of sense (filled bars) or antisense (hatched bars) oligodeoxynucleotides are shown and the data are presented as radioactive counts per arbitrary densitometric unit.

cells with oligodeoxynucleotides of similar base composition, generation of hydroxyl radicals and induction of DNA repair synthesis has been demonstrated (Iversen et al., unpublished data).

Data from flow cytometric and mitotic index analyses presented here indicate that the antisense oligodeoxynucleotides to annexin II and PGK retard cells in the S phase and this may indicate that PRP is necessary for traverse of S phase. This is consistent with the proposed function of PRP of enabling DNA polymerase α to synthesize DNA on the lagging-strand template at the replication fork (Pritchard and DePamphilis, 1983; Vishwanatha et al., 1986; Jindal and Vishwanatha, 1990a). Further support for the involvement of annexin II monomer in cell proliferation is provided by the identification of the growth-regulated gene 1B6 as annexin II monomer (Keutzer and Hirschhorn, 1990); increased expression of annexin II monomer in human hepatocellular carcinoma (Frohlich et al., 1990); enhanced expression of annexin II in pancreatic cancers of Syrian hamsters and association of annexin II with proliferating areas of the cancer (Kumble et al., 1992); and the loss of replicative ability in Xenopus laevis egg extracts immunodepleted with anti-annexin II antisera (Kumble, S. and Vishwanatha, J. K., in preparation). Experiments on new synthesis of PRP in the presence of antisense oligodeoxynucleotides indicate that the cellular level of PRP is not significantly affected by the antisense oligodeoxynucleotide, with a concomitant reduction in the newly synthesized PRP. This suggests that the newly synthesized annexin II monomer and PGK may be associated in the PRP complex. We have previously shown that PRP activity cannot be efficiently reconstituted by simple addition of purified PGK and annexin II monomer proteins (Jindal et al., 1991).

The data presented in this paper indicate a possible physiological role for annexin II as a PRP in DNA
synthesis and cell proliferation. We have presented the first evidence for the involvement of annexin II monomer in a cellular function.

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