Antiproliferative Activity of G-rich Oligonucleotides Correlates with Protein Binding*  

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Oligonucleotides have been extensively studied as antisense or antigenic agents that can potentially modulate the expression of specific genes. These strategies rely on sequence-specific hybridization of the oligonucleotide to mRNA or genomic DNA. Recently, it has become clear that oligonucleotides often have biological activities that cannot be attributed to their sequence-specific interactions with nucleic acids. Here we describe a series of guanosine-rich phosphodiester oligodeoxynucleotides that strongly inhibit proliferation in a number of human tumor cell lines. The presence of G-quartets in the active oligonucleotides is demonstrated using an UV melting technique. We show that G-rich oligonucleotides bind to a specific cellular protein and that the biological activity of the oligonucleotides correlates with binding to this protein. The G-rich oligonucleotide-binding protein was detected in both nuclear and cytoplasmic extracts and in proteins derived from the plasma membrane of cells. We present strong evidence that this protein is nucleolin, a multifunctional phosphoprotein whose levels are related to the rate of cell proliferation. Our results indicate that binding of G-rich oligonucleotides to nucleolin may be responsible for their non-sequence-specific effects. Furthermore, these oligonucleotides represent a new class of potentially therapeutic agents with a novel mechanism of action.

Oligonucleotides have the potential to recognize unique sequences of DNA or RNA with a remarkable degree of specificity. For this reason they have been considered as promising candidates to realize gene-specific therapies for the treatment of malignant, viral, and inflammatory diseases. Two major strategies of oligonucleotide-mediated therapeutic intervention have been developed, namely the antisense and antigenic approaches. The antisense strategy aims to down-regulate expression of a specific gene by hybridization of the oligonucleotide to the specific mRNA, resulting in inhibition of translation (1–4). The antigene strategy proposes to inhibit transcription of a target gene by means of triple helix formation between the oligonucleotide and specific sequences in the double-stranded genomic DNA (5). Clinical trials based on the antisense approach are now showing that oligonucleotides can be administered in a clinically relevant way and have few toxic side effects (1, 4).

Whereas both the antisense and antigenic strategies have met with some success, it has become clear in recent years that the interactions of oligonucleotides with the components of a living organism go far beyond sequence-specific hybridization with the target nucleic acid. Recent studies and reexamination of early antisense data have suggested that some of the observed biological effects of antisense oligonucleotides cannot be due entirely to Watson-Crick hybridization with the target mRNA. In some cases, the expected biological effect (e.g. inhibition of cell growth or apoptosis) was achieved, but this was not accompanied by a down-regulation of the target protein and was thus unlikely to be a true antisense effect (6, 7). In many cases, it was demonstrated that other non-sequence-specific oligonucleotides could exert biological effects that equaled or exceeded the antisense sequence (8–10). Although there is currently a high awareness among antisense investigators of the importance of appropriate control oligonucleotides, and the necessity of demonstrating inhibition of target protein production (11), the mechanism of non-antisense effects is poorly understood.

In particular, phosphodiester and phosphorothioate oligodeoxynucleotides containing contiguous guanosines (G) have been repeatedly found to have non-antisense effects on the growth of cells in culture (9, 10, 12). There is evidence that this activity is related to the ability of these oligonucleotides to form stable structures involving intramolecular or intermolecular G-quartets (9, 10). These are square planar arrangements of four hydrogen-bonded guanines that are stabilized by monovalent cations. Such structures are thought to play an important role in vivo, and putative quartet-forming sequences have been identified in telomeric DNA (13), immunoglobulin switch recombination sequences (14), human immunodeficiency virus, type I, RNA (15), the fragile X repeat sequences (16), and the retinoblastoma gene (17).

It has been suggested that non-antisense effects may be due to sequestration of intracellular or surface proteins by the oligonucleotide (18, 19). For G-rich oligonucleotides that can form folded or G-quartet-containing structures, this binding is not mediated by recognition of the primary sequence of the oligonucleotides but rather of their unique three-dimensional shape. However, the protein targets of these oligonucleotides have not been well characterized.

Here we identify a G-rich oligonucleotide-binding protein, and we show that the ability of G-rich oligonucleotides to bind to this protein is correlated with their propensity to form G-quartets, and with their ability to inhibit the growth of tumor cells.

EXPERIMENTAL PROCEDURES  

Oligonucleotides—3′-Modified oligonucleotides were purchased from Oligos Etc. (Wilsonville, OR) or synthesized at the University of Alabama at Birmingham using 3′-C3-amine CPG columns from Glen Research (Sterling, VA). Unmodified oligonucleotides were purchased from Life Technologies, Inc. Oligonucleotides were resuspended in water, precipitated with n-butyl alcohol, washed with 70% ethanol, dried,
and resuspended in sterile water or phosphate-buffered saline (PBS). They were then sterilized by filtration through a 0.2-μm filter. Each oligonucleotide was checked for integrity by 5'-radiolabeling followed by polyacrylamide gel electrophoresis. The results reported in this paper were reproducible and independent of the source of synthetic oligonucleotides.

Cells were plated at a density (10^4 to 10^5 cells per well, depending on cell line) in the appropriate serum-supplemented medium in 96-well plates. The following day (day 1) oligonucleotide (or water as control) was added to the culture medium to give a final concentration of 15 μM. On days 2–4 further oligonucleotide equivalent to half the initial dose was added. Cells were assayed using the MTT assay (20) on days 1, 3, 5, 7, and 9 after plating. The culture medium was not changed throughout the duration of the experiment (which was the time required for untreated cells to grow to confluence). Experiments were performed in triplicate, and bars represent the standard error of the data. For the experiments shown in Fig. 5, MDA-MB-231 breast cancer cells (5 × 10^4 cells per well) were plated in a 96-well plate. After 24 h, a single dose of oligonucleotide (or equal volume of PBS as a control) was added to the culture medium to a final concentration of 10 μM. Viable cells were assayed using the MTT assay 7 or 9 days (as indicated in the figure legend) after plating. For the experiment using 3'-unmodified oligonucleotides (Fig. 5D), serum-supplemented medium was replaced by serum-free medium containing oligonucleotide (or serum-free medium alone in control wells). After incubation for 48 h, fetal calf serum was added to the medium to give to 10% v/v. Heparin used in these experiments was USP grade sodium salt derived from porcine intestine, purchased from Apothecon (Bristol-Myers Squibb Co.). Working solutions were diluted from the stock (1000 units/ml) in sterile PBS.

Detection of G-quartets by UV Spectroscopy—Oligonucleotides were resuspended in Tmn buffer (20 mM Tris-HCl, pH 8.0, 140 mM KCl, 2.5 mM MgCl2) at a concentration such that A260 = 0.6 (molar concentrations ranged from 2.0 to 3.9 μM). Samples were annealed by boiling for 5 min and allowing to cool slowly to room temperature and overnight incubation at 4 °C. Thermal denaturation/renaturation experiments were carried out using an Amersham Pharmacia Biotech Ultraspec 2000 instrument equipped with a Peltier effect heated cuvette holder and temperature controller (Amersham Pharmacia Biotech). Absorbance at 295 nm was monitored over a temperature range of 25–95 or 20–90 °C and allowing to cool slowly to room temperature and overnight incubation. For the UV cross-linking experiments, samples described were 50% confluent in 90-mm dishes. The sequences of the oligonucleotides from prostate (DU145), breast (MDA-MB-231, MCF-7), or cervical (HeLa) carcinomas. The sequences of the oligonucleotides used in this and later experiments are shown in Table I. Two oligonucleotides, GRO29A and GRO15A, consistently inhibited proliferation in all of the cell lines tested. For three of the cell lines, GRO29A had a more potent inhibitory effect than

### Western Blotting

Western blotting was carried out at room temperature in PBS buffer containing Tween 20 at 0.1% v/v (for polyclonal antibody) or 0.05% (monoclonal antibody). PVDF membranes were blocked with PBS/Tween 20 containing 5% NDM for 1 h, washed, and incubated for 1 h with a 1:1000 dilution of nucleolin antisera or 1 μg/ml nucleolin monoclonal antibody (MBL Ltd., Japan) in PBS/Tween 20. The membranes were washed 3 times for 5 min with PBS/Tween 20 and incubated for 1 h with a 1:500 dilution of rabbit anti-HeLa IgG-horseradish peroxidase (1:2000 anti-mouse IgG-horseradish peroxidase). After washing as above the blot was visualized using ECL reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Capture of Biotinylated Oligonucleotide-Protein Complexes—MDA-MB-231 cells were grown to 50% confluence in 90-mm dishes. The cells were treated by addition of 5'-biotinylated oligonucleotide at a final concentration of 5 μM. After incubation for 2 h at 37 °C cells were washed extensively with PBS and lysed by addition of 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% (w/v) sodium azide, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.5 mM dithiothreitol, 1 mg/ml apronitin) to the cell suspension (Amersham Pharmacia Biotech) and allowed to cool slowly to room temperature and overnight incubation at 4 °C.

### Preparation of Nuclear, Cytoplasmic, and Membrane Protein Extracts—HeLa nuclear extracts used in EMSAs and Southwestern blotting were purchased from Promega Inc. (bandshift grade). Nuclear and cytoplasmic extracts were prepared from MDA-MB-231 cells using the protocol described in Ausbel et al. (21) Plasma membrane proteins were prepared from MDA-MB-231 cells using a method previously described for peroxisomes.

### Results

**Growth Inhibitory Effects of G-rich Oligonucleotides—**We tested the effects of four G-rich phosphodiester oligonucleotides (GROs) on the growth of tumor cells in culture. These oligonucleotides consisted entirely of deoxyguanosine and thymidine and contained runs of at least two contiguous guanosines. For increased stability to serum nucleases, oligonucleotides were modified at the 3'-terminus with a propyl amino group. We have observed previously that this modification protects oligonucleotides from degradation in serum-containing medium for at least 24 h.

Fig. 1 shows the results of MTT assays for determining relative numbers of viable cells in treated cell lines derived from prostate (DU145), breast (MDA-MB-231, MCF-7), or cervical (HeLa) carcinomas. The sequences of the oligonucleotides used in this and later experiments are shown in Table I. Two oligonucleotides, GRO29A and GRO15A, consistently inhibited proliferation in all of the cell lines tested. For three of the cell lines, GRO29A had a more potent inhibitory effect than

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1 The abbreviations used are: PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GRO, guanosine-rich oligonucleotide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDM, nonfat dried milk; PVDF, polyvinylidene difluoride; CRO, C-rich oligonucleotide.
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GRO15A (for MCF-7 cells, the oligonucleotides had similar effects). The growth of cells treated with two other oligonucleotides, GRO15B and GRO26A, was similar to that of the control water-treated cells (GRO26A had a weak growth inhibitory effect in MDA-MB-231 and HeLa cells). Examination of the growth-inhibited cells suggested that these effects may be cytostatic rather than cytotoxic. No indication of cell death (non-growth-inhibited cells) was observed when viewed using a phase contrast microscope (not shown).

**G-quartet Formation by G-rich Oligonucleotides**—To investigate the formation of G-quartet structures by the G-rich oligonucleotides, we used a UV melting technique described by Mergny et al. (24). This method relies on the fact that dissociation of G-quartets leads to a decrease in absorbance at 295 nm and is reported (24) to give a more reliable indication of intramolecular G-quartet formation than measurement at 260 nm. As a control for G-quartet formation, we used a single-stranded oligonucleotide, TEL. This oligonucleotide contains four repeats of the human telomere sequence 5’-TTAGGG and is known to form a G-quartet structure in vitro (25). Fig. 2 shows the annealing curve for this sequence. G-quartet formation is indicated by a clear transition with a melting temperature of 66 °C. The transition was reversible and a slight hysteresis was observed between heating and cooling curves (not shown) at 0.5 °C/min indicating a fairly slow transition. The most active oligonucleotide, GRO29A, showed a decrease in absorbance between 20 and 50 °C. This is suggestive of G-quartet formation, but a clear transition is not seen since the melting temperature is lower than for TEL or GRO29A. The curves for the two inactive oligonucleotides, GRO15A and GRO26A, showed no transitions characteristic of intramolecular G-quartet formation under these conditions.

**Relative Uptake of Oligonucleotides**—To determine if the antiproliferative activity of G-rich oligonucleotides could be explained by their differential uptake into cells, we assessed the cellular uptake of 5’-radiolabeled oligonucleotides. Although this method may underestimate absolute cellular uptake of oligonucleotide due to the action of phosphomonoesterase in removing the 5’-label, it can provide useful information when comparing relative uptake (26, 27). Fig. 3 shows the relative uptake of oligonucleotides into cells after 10 h, as measured by cell-associated radioactivity. The order of uptake (i.e., GRO15A > GRO29A > CRO > GRO15B > GRO26A > MIX1) was the same at 26 h. The presence of intact oligonucleotide inside cells was verified by polyacrylamide electrophoresis of cell lysates.

Although Fig. 3 shows that there were differences in the extent of oligonucleotide uptake depending on sequence, these did not correlate with antiproliferative activity. For example, an inactive oligonucleotide, CRO (see Fig. 5C), was taken up with similar efficiency to the most active oligonucleotide, GRO29A. Hence, the differential growth inhibitory properties of the oligonucleotides cannot be explained in terms of differences in cell uptake. We noted that relative uptake appeared to correlate well with the proportion (but not the number) of thymidines in the sequence, but the significance of this observation is not clear at present.
Active G-rich Oligonucleotides Bind to a Specific Cellular Protein—To investigate further the mechanism of the growth inhibitory effects, we examined binding of the oligonucleotides to cellular proteins. 5'-Radiolabeled oligonucleotides were incubated with HeLa nuclear extracts, alone or in the presence of unlabeled competitor oligonucleotide, and examined by an electrophoretic mobility shift assay. The G-quartet forming telomere sequence oligonucleotide, TEL, was also included as a competitor in this experiment. Fig. 4A shows the formation of a stable protein-oligonucleotide complex (Fig. 4A, *). This band was intense when the labeled oligonucleotide was one of the growth inhibitory oligonucleotides, GRO15A or GRO29A (lanes 1 and 5), but the inactive oligonucleotide, GRO26A, formed only a weak complex (lane 9). This experiment also showed that the complex could be effectively competed by either unlabeled antiproliferative oligonucleotide or TEL but not by the inactive GRO26A.

To confirm further that the same protein is binding to TEL and to the growth inhibitory oligonucleotides, we carried out a similar experiment in which TEL was labeled. Labeled TEL formed two complexes with nuclear extracts in the absence of competitor oligonucleotides (bands A and B, Fig. 4B). The slower migrating TEL-protein complex (band A) was competed for by unlabeled growth inhibitory oligonucleotides (GRO15A and GRO29A) but not inactive oligonucleotides (GRO26A and GRO15B). The faster migrating complex (band B) was specific for TEL and was not competed for by G-rich oligonucleotides. Hence binding of competitor GROs was characterized by a decrease in the intensity of band A and an increase in the intensity of band B (due to release of labeled TEL from band A complex). This assay allowed comparison of the binding affinity of native GROs (without 5'-phosphorylation) and was used for assessment of protein binding in subsequent experiments. To ensure that competition was due to binding of the GRO to the protein component of complex A, and not a result of interaction between GRO and TEL oligonucleotide, we carried out a mobility shift on a 15% polyacrylamide gel. No shifted bands were observed when labeled TEL was incubated with GROs in the absence of protein (data not shown).

To determine the approximate molecular weight of the pro-
tein involved in complex A, and to confirm direct binding of the protein to oligonucleotides, we carried out a UV cross-linking study. 5'-Labeled oligonucleotides and HeLa nuclear extracts were incubated alone or in the presence of unlabeled competitor oligonucleotides. The samples were then irradiated with UV light resulting in cross-link formation between protein residues and thymidines in the oligonucleotide. The protein was thus radiolabeled and could be detected on an SDS-polyacrylamide gel. Fig. 4C shows the results of this experiment. Both TEL and GRO15A cross-linked to a protein (Fig. 4C, *) which was competed for by antiproliferative oligonucleotides and TEL but not by inactive GRO26A. The most active oligonucleotide, GRO29A, also formed this approximately 100-kDa complex and another complex of higher molecular weight (not shown). Inactive GRO26A produced a barely visible band at ~100 kDa (not shown).

The molecular weight of the nuclear protein was more accurately determined by Southwestern blotting. HeLa nuclear extracts were electrophoresed on an 8% polyacrylamide-SDS gel and transferred to a PVDF membrane. The membrane was blocked and cut into strips. Each strip was incubated at 4°C with a 32P-labeled G-rich oligonucleotide in the presence of unrelated unlabeled double-stranded and single-stranded DNA to block nonspecific binding. Fig. 4D shows active oligonucleotides GRO15A and GRO29A hybridized to a single protein band at 106 kDa (the band was exactly adjacent to a 106-kDa molecular mass marker, not shown). Inactive oligonucleotides GRO15B and GRO26A hybridized only weakly to this protein. The data presented in Figs. 1 and 4 suggest a correlation between activity and protein binding, at least for the four oligonucleotides examined. These experiments also demonstrate that binding of GROs to p106 is highly specific, since only a single protein band is recognized with high affinity (see Fig. 4D). This was not simply a result of hybridization to an abundant protein, as India ink staining of immobilized nuclear extracts showed the presence of many other protein bands that were equally or more intense than the band at 106 kDa (data not shown).

Antiproliferative Activity of G-rich Oligonucleotides Correlates with Protein Binding—To confirm further the relationship between activity and binding to the 106-kDa protein, we synthesized four more G-rich oligonucleotides and compared their effects with active (GRO29A) and inactive (GRO15B) oligonucleotides. Fig. 5, A and B, shows that the growth inhibitory effect of the oligonucleotides correlated with their ability to compete for the TEL-binding protein. Three of the new oligonucleotides displayed a moderate antiproliferative activity but were not as potent as GRO29A. Oligonucleotide GRO14B showed no antiproliferative activity. Correspondingly, the moderately active oligonucleotides (GRO14A, GRO25A, and GRO28A) were able to compete with TEL for binding to the nuclear protein, although not as effectively as GRO29A. The non-inhibitory oligonucleotide, GRO14B, was unable to compete for protein binding.

Effects of Non-G-rich Oligonucleotides—To investigate the specificity of the antiproliferative effects, we examined the growth inhibitory effects of non-G-rich oligonucleotides and heparin, a polyanionic polysaccharide. Fig. 5C shows that at 10 μM concentration (equivalent to approximately 0.1 mg/ml for GRO29A), neither a 3'-modified G-rich oligonucleotide (CRO) nor a 3'-modified mixed base oligonucleotide (MIX1) were able to inhibit the growth of MDA-MB-231 breast cancer cells. This result showed that the growth inhibiting activity of GRO15A and GRO29A was not simply nonspecific effects resulting from the presence of 3'-modified oligonucleotide but rather relied on some unique feature of these sequences. Heparin also had no effect on cell growth when added to the culture medium at a concentration of 20 units/ml (approximately 0.12 mg/ml), further demonstrating that the antiproliferative effects of active oligonucleotides are not simply a result of their polyanionic character. To examine the antiproliferative properties of non-3'-protected oligonucleotides, we used a slightly modified treatment protocol in which oligonucleotides were added to cells in serum-free medium (see “Experimental Procedures”). Fig. 5D shows that similar effects could also be seen with unmodified oligonucleotides under these conditions. Both 29A-OH (a 3’-unmodified analog of GRO29A) and TEL inhibited the growth of cells, whereas two mixed sequence oligonucleotides had no growth inhibitory effects.

We also compared the protein binding properties of these non-G-rich oligonucleotides and heparin (not shown). As expected, the unlabeled growth inhibitory oligonucleotides GRO29A, 29A-OH, and TEL competed strongly for protein binding in the competitive electrophoretic mobility shift assay (using labeled TEL oligonucleotide and MDA-MB-231 nuclear extracts) at 10 nM concentration (approximately 0.1 μg/ml for GRO29A). In accord with its lesser antiproliferative activity, TEL competed slightly less effectively than 29A-OH or GRO29A. No competition was observed using 10 nM unlabeled CRO, MIX2, or MIX3 in the presence of 0.02 units/ml heparin (approximately 0.12 μg/ml). However, the mixed sequence oligonucleotide, MIX1, was anomalous. Although this oligonucleotide had no effect on the growth of cells, it appeared to compete for protein binding in the competitive EMSA.

Evidence That G-rich Oligonucleotide-binding Protein Is Nucleolin—Two previous reports describe binding of the nuclear protein, nucleolin, to the G-rich telomere sequence. Ishikawa et al. (28) identified a 50-kDa protein from HeLa extracts that bound to 5’-(TTAGGG)4-3’. Microsequence determination suggested that this was a proteolytic fragment of nucleolin. Binding of the full-length, purified 106-kDa nucleolin protein was demonstrated independently by Dickinson and Kohwi-Shigematsu (29). Since our protein was of the correct molecular weight and also bound to 5’-(TTAGGG)4-3’ (TEL), we tested the hypothesis that the G-rich oligonucleotide-binding protein was nucleolin. Nuclear extracts from HeLa cells (purchased from Promega) or MDA-MB-231 breast cancer cells (obtained in our laboratory by standard procedures) were electrophoresed and transferred to PVDF membrane. The immobilized proteins were probed for binding to 32P-labeled GRO15A using the
Southwestern procedure described and were visualized by overnight exposure to autoradiographic film. The same membrane was stripped of oligonucleotide by the denaturation/re-naturation steps described (see “Southwestern Blotting” under “Experimental Procedures”) and Western-blotted using nucleolin antiserum as primary antibody and a horseradish peroxidase-conjugated anti-rabbit secondary antibody. The blot was visualized by incubation with a chemiluminescence detection reagent followed by a 20-s exposure to autoradiographic film. The results are shown in Fig. 6A. Southwestern blots of nuclear extracts showed an intense band upon hybridization with radiolabeled GRO15A at 106 kDa (HeLa) or 116 kDa (MDA-MB-231). The Western blot of MDA-MB-231 nuclear proteins shows one intense band at 116 kDa and weaker bands at about 50 kDa. In HeLa extracts the nucleolin antibody recognizes multiple bands at approximately 50, 75, 106, and 120 kDa. Most importantly, in both cell lines the band that was recognized by GRO15A exactly corresponded to a band recognized when the membrane was stripped and Western-blotted with nucleolin antibody. Nucleolin is a protein that can be phosphorylated in cells by a number of kinases and is also highly susceptible to proteolysis (30–36). We believe that the difference in the molecular weight of proteins detected in these blots may arise from the different methods of preparation of the nuclear extract leading to differently phosphorylated or degraded forms of nucleolin being the predominant species. The difference in the intensities of the bands shown in the Southwestern blots in Fig. 6A may be due to the preferential binding of GRO15A to one form of nucleolin (apparently the 106-kDa species) over others.

To determine whether binding of the specific protein occurred within the cell, we used biotinylated G-rich oligonucleotides to treat MDA-MB-231 breast cancer cells. Streptavidin-coated magnetic beads were then used to capture oligonucleotide–protein complexes after lysing the cells with an immunoprecipitation-type buffer (see “Experimental Procedures”). This procedure was carried out for cells that were treated with either an active oligonucleotide (5'-Biotin-GRO15A) or an inactive oligonucleotide (5'-Biotin-GRO15B) and untreated cells as a control. Equal volumes of each sample were electrophoresed and transferred to a PVDF membrane. This was analyzed by india ink staining, Southwestern blotting with radiolabeled GRO15A, and Western blotting with a nucleolin monoclonal antibody. India ink staining of the membrane showed a major protein band at 116 kDa that was present in cells treated with biotinylated GRO15A but was absent in untreated cells and of a lower intensity in cells treated with inactive biotinylated GRO15B (data not shown). The Southwestern and Western blots (Fig. 6B) confirm that this captured protein binds to both GRO15A and a nucleolin antibody. This experiment showed that a 116-kDa protein was specif-
complex formed by binding of 5 μg of MDA-MB-231 nuclear extracts to 32P-labeled TEL oligonucleotide and competition by unlabeled G-rich oligonucleotides (10-fold molar excess). C, MTT assay of MDA-MB-231 cells treated with a single 10 μM dose of 3’-protected C-rich oligonucleotide (CRO) or mixed sequence oligonucleotide (MIX1) or with 20 units/ml heparin (HEP), in comparison with inactive (GRO15B) and active (GRO28A) G-rich oligonucleotides. The assay was performed on day 9 (oligonucleotide added on day 1). B, EMSA showing complex formed by binding of 5 μg of MDA-MB-231 nuclear extracts to a GRO-binding protein in the plasma membrane showed that a monoclonal antibody to nucleolin also hybridized strongly to GRO15A. Western blotting of the same membrane extracts also suggests the possibility that binding to cell surface protein may be important in the mechanism of action of G-rich oligonucleotides.

**DISCUSSION**

Oligonucleotides are polyanionic species that are internalized in cells, probably by receptor-mediated endocytosis (37). They are likely to interact with many biomolecules within the cell and also in the extracellular membrane by virtue of both their charge and their shape, as well as sequence-specific interactions with nucleic acids. The proteins that bind to oligonucleotides and mediate non-antisense effects have not yet been unequivocally identified.

We have described G-rich oligonucleotides that have potent growth inhibitory effects that are unrelated to any expected antisense or antigen activity. Although we have not yet delineated the mechanism of these effects, we have demonstrated that the antiproliferative effects of these oligonucleotides are related to their ability to bind to a specific cellular protein. Because the GRO-binding protein is also recognized by antinucleolin antibodies, we conclude that this protein is either
nucleolin itself or a protein of a similar size that shares immunogenic similarities with nucleolin.

Nucleolin is an abundant multifunctional 110-kDa phosphoprotein, thought to be located predominantly in the nucleolus of proliferating cells (for reviews, see Refs. 38 and 39). It has been implicated in many aspects of ribosome biogenesis including the control of rDNA transcription, pre-ribosome packaging, and organization of nucleolar chromatin (38–40). Another emerging role for nucleolin is as a shuttle protein that transports viral and cellular proteins between the cytoplasm and nucleus/nucleolus of the cell (41–43). Nucleolin is also implicated, directly or indirectly, in other roles including nuclear matrix structure (44), cytokinesis, and nuclear division (45) and as an RNA and DNA helicase (46). Its multifunctional nature is reflected in its multidomain structure, consisting of a histone-like N terminus, a central domain containing RNA recognition motifs, and a glycine- and arginine-rich C terminus (47). Levels of nucleolin are known to relate to the rate of cellular proliferation (48, 49), being elevated in rapidly proliferating cells, such as malignant cells, and lower in more slowly dividing cells. For this reason, nucleolin may be an attractive therapeutic target for the treatment of malignant disease.

Although considered a predominantly nucleolar protein, our finding that nucleolin was present in the plasma membrane is consistent with several reports identifying cell surface nucleolin and suggesting its role as a cell surface receptor (50–53). Previously, several mechanisms have been proposed to explain the non-sequence-specific effects of oligonucleotides. These include binding to cellular receptors (54, 55), modulation of cytokine or growth factor activity (56–60), inhibition of cell proliferation (51), and suggesting its role as a cell surface receptor (50–53).

In this present report, we have identified nucleolin (or a nucleolin-like protein) as a G-rich oligonucleotide-binding protein, and we have shown a strong correlation between binding to this protein and antiproliferative activity for a series of G-rich oligonucleotides. We believe that these findings strongly suggest a mechanistic role for nucleolin in non-antisense inhibition of cell growth by G-rich oligonucleotides. This belief has been strengthened by our recent immunofluorescence experiments that show significant differences in nucleolin levels between cells treated with GRO29A and untreated cells.2

The relationship between nucleolin binding and antiproliferative activity for other, non-G-rich, oligonucleotides has not yet been fully evaluated. One mixed sequence oligonucleotide (MIX1) was found to bind to nucleolin, although it had no growth inhibitory effect. Nucleolin contains RNA binding domains that can recognize specific sequences of RNA or single-stranded DNA (29, 61). It is possible that this particular oligonucleotide contains a sequence or structure that resembles such a recognition element.

In support of our findings that nucleolin binds selectively to G-rich oligonucleotides that form stable G-quartet structures, Maizels et al. (62, 63) have recently demonstrated binding of purified nucleolin to G-quartet forming DNA sequences from immunoglobulin switch regions and ribosomal DNA. It is likely that nucleolin has currently undefined functions in vivo that depend on recognition of G-quartet forming sequences in ribosomal DNA, switch region sequences, or telomeres.

It is our hypothesis that nucleolin contains a specific binding site that recognizes certain G-quartet structures and that binding at this site by a G-rich oligonucleotide inhibits one or more of the normal functions of nucleolin. The consequences of nucleolin inhibition on the growth of cells have not been well studied, but it is easy to envisage that inhibition of a protein whose functions include ribosome production, nuclear transport, and cell entry could have profound effects on the growth of cells.

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