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Phosphorothioate oligonucleotides bind in a non sequence-specific manner to the nucleolar protein C23/nucleolin

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Abstract **To design optimal strategies for intracellular delivery of antisense phosphorothioate oligonucleotides, it may be useful to understand their interaction with cellular macromolecules. Nuclear extracts from LOX amelanotic myeloma cells were studied for protein binding to phosphorothioate oligonucleotides using a Southwestern protocol. Multiple nuclear proteins bound to the phosphorothioate oligonucleotides but no detectable protein binding was found to phosphodiester oligonucleotides. The protein with the strongest binding signals was shown by immunoprecipitation to be nucleolar C23/nucleolin, a 110 kDa protein. With glutathione S-transferase/nucleolin fusion protein constructs, the region of nucleolin containing the RNA recognition motifs had binding activity to phosphorothioate oligonucleotides.**

Key words: Phosphorothioate oligonucleotide; C23/nucleolin; RNA recognition motif

1. Introduction

Antisense oligonucleotides have been shown to inhibit gene expression in a variety of in vitro systems [1–5]. Design of optimal strategies for delivery of antisense oligonucleotides requires an understanding of the mechanisms of internalization into cells and their subcellular localization. Studies have shown that internalization of some antisense oligonucleotides occurs by pinocytosis, which involves a 75–80 kDa membrane receptor protein [6–8]. Other studies have explored the intracellular trafficking and the intracellular macromolecules to which antisense oligonucleotides bind. Some antisense oligonucleotides were shown to accumulate in nuclei [8–13] and bind to nuclear proteins [8,10,14], but no specific nuclear binding protein was found.

Phosphorothioate oligonucleotides are being evaluated as inhibitors of gene expression: they have the advantage of greater stability than phosphodiester oligonucleotides because of their greater resistance to nucleases [15]. Stein et al. [15] reported one to two order of magnitude increased non-sequence-specific protein binding by phosphorothioate oligonucleotides as compared to phosphodiester oligonucleotides. Such interactions could cause non-sequence-specific biological effects, and may affect the subcellular localization and the antisense activity of phosphorothioate oligonucleotides.

Using nuclear protein extracts from human LOX amelanotic myeloma culture cells and an in vitro Southwest protein binding assay, we have demonstrated several nuclear proteins bound phosphorothioate oligonucleotides in a non-sequence specific manner. These proteins did not bind phosphodiester oligonucleotides. The protein with the highest binding signal to phosphorothioate oligonucleotides has been identified by immunoprecipitation as C23/nucleolin. Using bacterially expressed recombinant glutathione S-transferase/nucleolin fusion proteins, binding of phosphorothioate oligonucleotides to nucleolin was localized to the carboxy half of the protein, the region which contains the RNA recognition motifs of nucleolin.

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Abbreviations: GST, glutathione S-transferase; RRM, RNA recognition motif.

2. Materials and methods

2.1. Antisense oligonucleotides

Synthesis of antisense phosphorothioate oligonucleotides to the p120 gene sequence has been previously described [16]. The sequences are: ISIS 3466-CACCCGCCTTGGCCTCCCAC; ISIS 3790-GTGGGAGGCCAAGGCGGGTG; ISIS 3460-CCCCATGGTACTGTGGCAGG; ISIS 3462-CCTTCCTCCCGCTGAGCCCC; ISIS 3789-CACGCCTCCCGACTCTGCCC.

BV-102 (phosphodiester analog of ISIS 3466), phosphodiester ologonucleotides BV-90 (AGGAGACTTATTTGGCTTAGG) and BV-96 (CATGGGGCGCAAGTTCAATCC) were synthesized by the Department of Cell Biology, Baylor College of Medicine. RE7, a random sequence phosphorothioate oligonucleotide with sequence TATTACGTACTAGATTCTAC has been previously described [17].

2.2. Cell lines

Human amelanotic melanoma cells (LOX), provided by Dr. D.J. Dykes, (Southern Reseach Institute, Birmingham, AL) were subcultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin liquid (Gibco BRL).

2.3. Preparation of nuclear and cytoplasmic extract

LOX nuclear and cytoplasmic protein extracts were prepared essentially as described by Dignam et al. [18]. Protein concentration was determined by the Bradford assay.

2.4. SDS-PAGE, Southwestern, and Western blot analysis

Protein extracts were electrophoresed on SDS-polyacrylamide gels as described by Laemmli [19], and proteins transferred to nitrocellulose electrophoretically at 100 V for one hour in transfer buffer (192 mM glycine, 25 mM Tris-HCl, 20% (v/v) methanol). Southwestern analysis was modified from Miskimins et al. [20]. Blots were blocked in 5% nonfat dry milk in 10 mM HEPES, pH 7.9, for at least one hour. Binding to radiolabeled phosphorothioate oligonucleotides was performed in binding buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.25% nonfat dry milk, 5 μg/ml poly I/poly C) containing 1×10^5 CPM/ml of ^{32}P oligonucleotide labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding was done for a minimum of 1.5 h at room temperature. The blots were washed for 2 h in 2 changes of wash buffer (same as binding buffer including 0.3 M NaCl and without poly I/ poly C or labeled oligonucleotide). The blot was covered with plastic wrap and exposed to autoradiography using an intensifying screen at −70°C.

For Western analysis, blots were blocked in 5% nonfat dry milk in 10 mM HEPES, pH 7.9. Binding to anti-C23 monoclonal antibody MS3 [21] was done at a 1:400 dilution of ascites in TBST buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween-20) for 2 h followed by three washes of 5 min in TBST. Binding to alkaline phosphatase-

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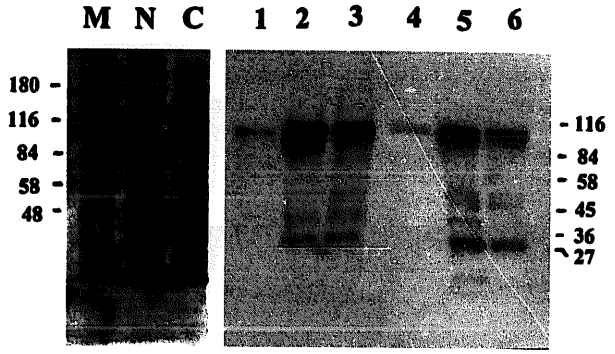

Fig. 1. Detection of phosphorothioate oligonucleotide binding to LOX cell nuclear and cytoplasmic protein extracts. LOX cell nuclear extracts from two independent preparations (lanes 2–3 and 5–6) and cytoplasmic extract (lanes 1 and 4) were separated on a 4–15% SDS polyacrylamide gradient gel and transferred to nitrocellulose filters. The filters were incubated with 5'-^{32}P-labeled ISIS 3790 (lanes 1–3) or ISIS 3466 (lanes 4–6) as described in section 2, and exposed to autoradiography. The position of prestained molecular mass markers for the Southwest blots are indicated to the right. The left three lanes show Coomassie blue staining of the protein extracts: M, molecular weight markers; N, nuclear extract; C, cytoplasmic extract.

conjugated secondary antibody (goat anti-mouse IgG from Promega) was done for 1 h followed by 3 washes in TBST. Color development was done with alkaline phosphatase reagents according to the Promega protocol.

2.5. Immunoprecipitation of nucleolin

LOX nuclear extract (50 μg) was precleared with Protein G Plus/Protein A agarose affinity beads (Oncogene Science) in IP buffer (10 mM Tris HCl (pH 8.0), 10 mM KCl, 0.5 mM $MgCl_2$ containing 1 μg/ml each leupeptin, pepstatin, and aprotinin) for one h at 4°C, and then precipitated with anti-nucleolin monoclonal antibody MS3 (20 μg purified IgG), overnight at 4°C in the same buffer. The precipitated complexes were tumbled with Protein G Plus/Protein A agarose beads for 6 h at 4°C, and washed three times in IP buffer. The washed beads containing the precipitated complexes were resuspended in SDS gel loading buffer (100 mM Tris-HCl, pH 6.8, 0.2 M dithiothreitol, 4% SDS, 20% glycerol), boiled for five min, and electrophoresed on 4–15% polyacrylamide gels. Transfer to nitrocellulose and binding to radiolabeled oligonucleotides were as described above.

2.6. Construction of nucleolin deletion mutants

All deletion mutants were constructed and expressed as glutathione S-transferase (GST) fusion proteins in DH5alpha *E. coli* cells. Plasmid pMAMC23, a pMAMneo construct (Clontech) containing the human nucleolin cDNA [22], was digested with *Msc*I and *Xho*I and treated with Klenow fragment of DNA polymerase I. The nucleolin cDNA fragment was agarose-gel purified using the QIAquick gel extraction kit (Qiagen) and ligated with blunt-ended *Eco*RI-digested pGEX 3X vector (Pharmacia) which resulted to clone pGC23/3'. The orientation of the insert was checked by *Bam*HI and *Nco*I digestion. DNA sequencing was done to confirm if nucleolin cDNA was in frame with the GST sequence.

Deletion from the 3' end of nucleolin was done by *Nco*I digestion of pGC23/3' and filling in the 5' protruding end with alpha-phosphorothioate nucleotides and Klenow fragment of DNA polymerase I. The DNA was then digested with *Hin*dIII and 3' to 5' deletion was done using Erase-a-Base kit (Promega).

A similar procedure was done for 5' deletion. Plasmid pGC23/3' was linearized with *Bam*HI and the 5' protruding end was protected. The linearized plasmid was digested with *Sma*I prior to *Exo*III digestion using Erase-a-Base kit. All deletion mutants were sequenced using the Sequenase kit (Amersham Life Science).

The GST-p120 construct (pG116) was prepared as described previously [23].

2.7. Expression and purification of GST fusion proteins

An overnight 5.0 ml Luria broth culture was added into 50 ml Luria broth with 100 μg/ml ampicillin. After 1 h of shaking at 37°C, expression was induced with 0.5 mM IPTG for 3 h. The *E. coli* cells were pelleted and resuspended in 5 ml NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1 mM PMSF, 0.25 mg/ml *N*-tosyl-phenylalanyl-chloromethyl ketone). The cells were lysed by mild sonication on ice and centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was tumbled for 30 min at 4°C with 0.15 ml of glutathione-Sepharose 4B (Pharmacia) which had been previously washed three times and resuspended (50% resin) in NETN buffer. The slurry was centrifuged at 500 × *g* for 5 min at 4°C and the pellet was washed 3 times with NETN buffer. The resin was boiled for 5 min in 0.15 ml sample buffer (0.126 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.001% Bromophenol blue) prior to gel electrophoresis.

3. Results

3.1. Phosphorothioate oligonucleotides bind efficiently to a 110 kDa nuclear protein from LOX cells in a non-sequence specific manner

Several phosphorothioate oligonucleotides which were synthesized as antisense oligonucleotides to the gene encoding p120 protein [16], a human proliferation-associated nucleolar antigen, were assayed for LOX nuclear protein binding by a Southwestern method. As shown in Fig. 1, strong binding to an approximately 110 kDa protein was detected with phosphorothioate oligonucleotides ISIS 3466 and its complementary sequence ISIS 3790. Minor binding was also observed to four 25–50 kDa proteins. Other phosphorothioate oligonucleotides, including ISIS 3460, ISIS 3462, ISIS 3789, and RE7, (see section 2) showed similar strong binding to a 110 kDa nuclear protein, which was the most dense band detected for all phosphorothioate oligonucleotides tested (data not shown). The

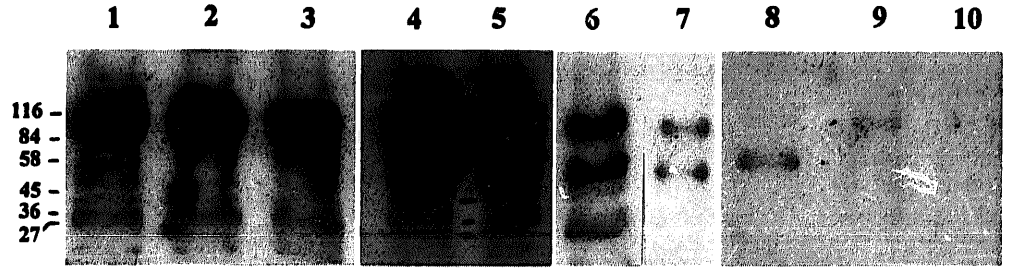

Fig. 2. Competition with unlabeled oligonucleotides: 5'-^{32}P-labeled ISIS 3466 (1 pmol) was incubated with nitrocellulose filters containing LOX cell nuclear extract as described in A. The following unlabeled competitor oligonucleotides were included in the incubation: Lane 1-none; lane 2-phosphodiester oligonucleotide BV 102, 100 pmol; lane 3- BV 102, 1,000 pmol; lane 4- phosphodiester oligonucleotide BV 90, 1,000 pmol; lane 5-phosphodiester oligonucleotide BV 96, 1,000 pmol; lane 6, phosphorothioate oligonucleotide RE7, 10 pmol; lane 7, RE7, 100 pmol; lane 8, RE7, 1,000 pmol; lane 9, phosphorothioate oligonucleotide ISIS 3466, 100 pmol; lane 10, phosphorothioate oligonucleotide ISIS 3462, 100 pmol.

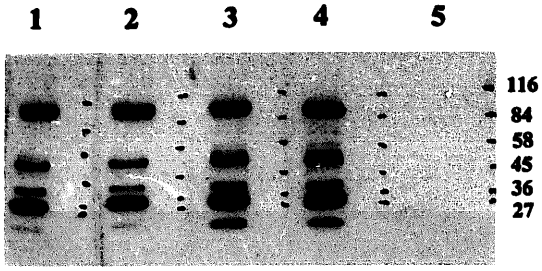

Fig. 3. Competition with polysaccharides. 5'-^{32}P-labeled ISIS 3466 (1 pmol) was incubated with nitrocellulose filters containing LOX cell nuclear extract. The following polysaccharides or oligonucleotide were tested for competition with binding of ISIS 3466, all at 1,000 pmol (1,000-fold molar excess): Lane 1, no added competitor; lane 2, dextran (mol.wt. 70,000); lane 3, dextran sulfate (mol.wt. 8,000); lane 4, pentosan polysulfate (mol.wt. 3,000); lane 5, unlabeled ISIS 3466.

110 kDa phosphorothioate oligonucleotide-binding protein was also detected in the cytoplasmic fraction, but at a much lower level in comparison to the nuclear fraction (Fig. 1). No LOX cell nuclear protein binding was detected using ^{32}P-labeled phosphodiester oligonucleotide BV-102, which is homologous to ISIS 3466 (data not shown).

To verify that the binding to the 110 kDa protein was sequence non-specific and was dependent on the presence of phosphorothioate in the oligonucleotide, competition studies were performed. Using ^{32}P-labeled phosphorothioate oligonucleotide ISIS 3466 and various unlabeled competing oligonucleotides, all phosphorothioate oligonucleotides tested (ISIS 3466, ISIS 3462, and RE7) efficiently competed with the binding of the labeled phosphorothioate oligonucleotide to the 110 kDa protein (Fig. 2, lanes 6–10). The phosphodiester oligonucleotides tested (BV-102, BV-90, and BV-96) did not compete with the binding by ISIS 3466 to the 110 kDa protein (lanes 2–5). No degradation of either phosphodiester or phosphorothioate oligonucleotides during the competition binding reaction was detected, by polyacrylamide gel electrophoresis (data not shown). Accordingly, phosphorothioate oligonucleotides but not phosphodiester oligonucleotides bind predominantly to the 110 kDa protein.

Phosphorothioate oligonucleotides are polyanionic compounds. Some proteins have a general affinity for polyanionic compounds, such as that of the human immunodeficiency virus protein gp120, whose V3 loop binds sulfated polysaccharides as well as phosphorothioate oligonucleotides [24]. To test if other polyanionic compounds competed with binding of the phosphorothioate oligonucleotide ISIS 3466 to nuclear proteins detected in the Southwestern assay, dextran sulfate, pentosan polysulfate, or unsulfated dextran (nonanionic polymer control) were used. At a 1,000-fold molar excess these polysaccharides were unable to compete with binding of ISIS 3466 to the nuclear proteins (Fig. 3, lanes 2–4). A 1,000-fold excess unlabeled ISIS 3466 completely competed with the binding of radiolabeled 3466 (lane 5). The binding of phosphorothioate oligonucleotides to these nuclear proteins is clearly not the result of general binding to polyanions.

3.2. *The 110 kDa phosphorothioate oligonucleotide binding protein is nucleolin*

By Southwestern analysis, the 110 kDa phosphorothioate oligonucleotide binding protein was enriched in nucleolar extracts (data not shown). To identify nucleolar proteins which comigrated with the 110 kDa oligonucleotide binding protein, monoclonal antibodies to nucleolar proteins were used. The 110 kDa phosphorothioate oligonucleotide binding protein comigrated precisely with C23/nucleolin, as detected by Western blot analysis (see Fig. 4, lanes 1 and 4). To determine if the 110 kDa phosphorothioate oligonucleotide binding protein was C23/nucleolin, it was immunoprecipitated from LOX nuclear extracts by anti C23/nucleolin antibodies. Western blot analysis confirmed that the immunoprecipitated protein was in fact nucleolin (Fig. 4, lower panel). The immunoprecipitated C23/nucleolin bound to ^{32}P-labeled phosphorothioate oligonucleotides ISIS 3466 and ISIS 3790, (Fig. 4, upper panel).

3.3. *Localization of the nucleolin phosphorothioate oligonucleotide binding region*

To determine the regions of nucleolin that bound to the phosphorothioate oligonucleotides, various fragments of nucleolin cDNA were fused with the glutathione S-transferase gene. The fusion proteins were purified from *E. coli*, and South-

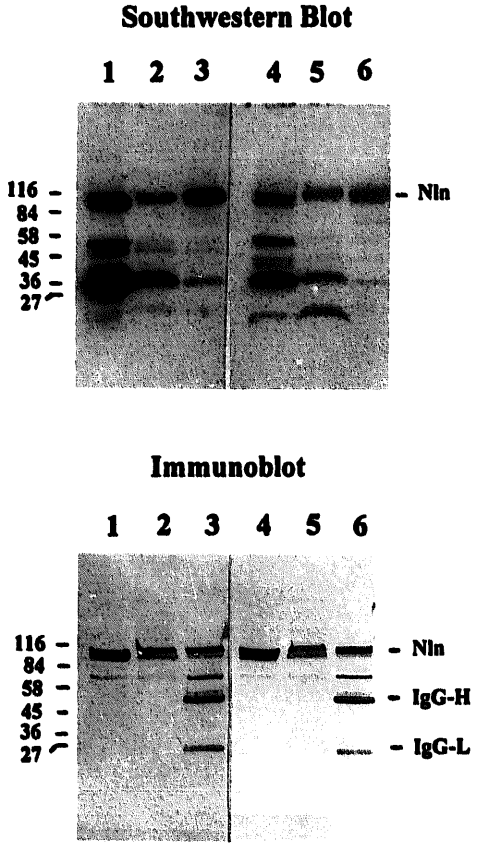

Fig. 4. Phosphorothioate-oligonucleotide binding to immunoprecipitated nucleolin.Nucleolin was immunoprecipitated from LOX nuclear extracts as described in section 2. Protein fractions were electrophoresed on 4–15% SDS polyacrylamide gradient gels, transfered to nitrocellulose, and incubated with 5'-^{32}P-labeled ISIS 3466 (lanes 1–3) or ISIS 3790 (lanes 4–6). Lanes 1 and 4, untreated LOX nuclear extracts; lanes 2 and 5, supernatant from LOX nuclear extracts immunoprecipitated with nucleolin monoclonal antibody MS3; lanes 3 and 6, precipitate from LOX nuclear extracts immunoprecipitated with nucleolin monoclonal antibody MS3. Nln, nucleolin; IgG-H, immunoglobulin G, heavy chain; IgG-L, immunoglobulin G, light chain.

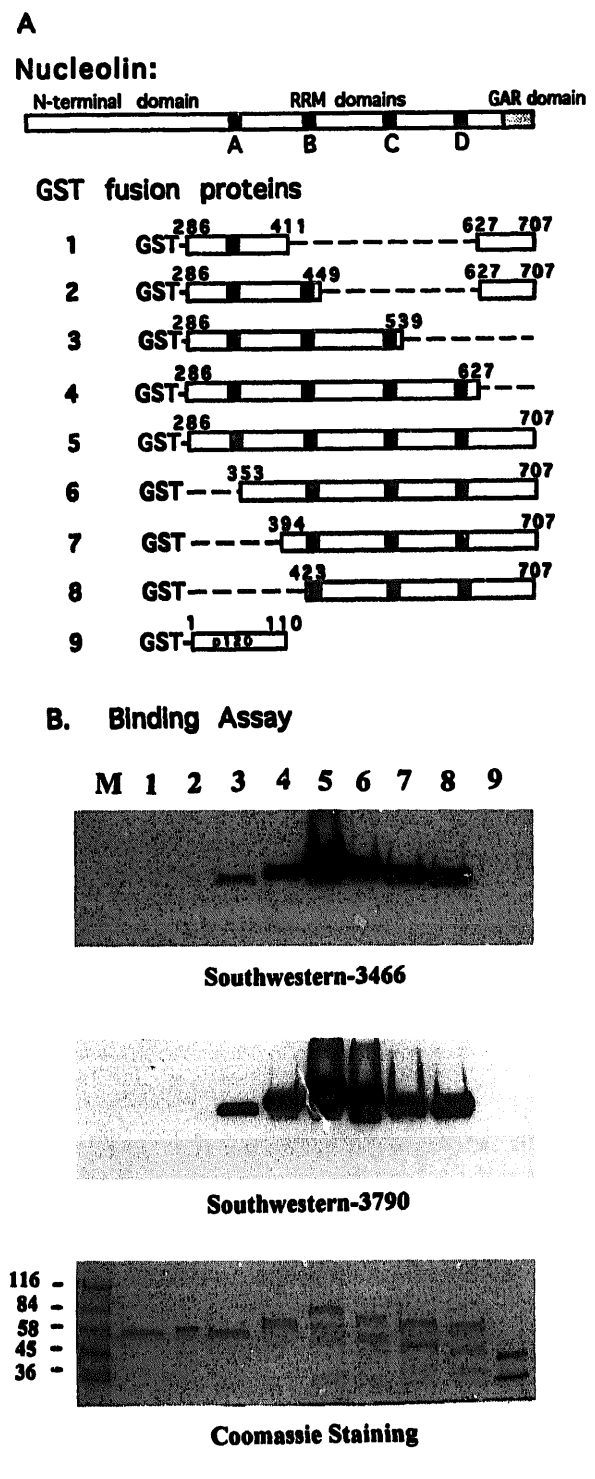

Fig. 5. Glutathione S-transferase/nucleolin fusion proteins (A) and their binding to phosphorothioate oligonucleotides (B).The fusion proteins were prepared and purified as described in section 2. Approximately equal amount of fusion proteins, as determined by Coomassie blue staining, were electrophoresed, transferred to nitrocellulose, and bound with 2 pmol of ^{32}P-labeled ISIS 3466 (upper panel) or ISIS 3790 (middle panel). The open rectangles (clones 1–8) represent the nucleolin sequence with numbers indicating the positions of amino acid residues and broken lines represent the deleted nucleolin sequences. Each hatched box represents the 11-amino acid consensus sequence for RNA recognition. Clone 9 is a fusion of GST and amino acids 1–110 of nucleolar protein p120. Lane numbers in (B) correspond to clone numbers in (A).

western analysis was done with ^{32}P-labeled ISIS 3466 or ISIS 3790. No binding of either phosphorothioate oligonucleotide to a control fusion protein GST/p120 was detected (Fig. 5, lane 9). Binding was observed for GST/nucleolin fusion proteins containing nucleolin sequences 286–539, 286–627, 286–707, 353–707, 394–707, and 423–707 (Fig. 5, lanes 3–8). No binding was detected to nucleolin fragments containing sequences from 286–411 or 286–449 (Fig. 5, lanes 1 and 2; both contain amino acids 627–707). Coomassie staining showed that similar amounts of fusion proteins were loaded in each lane (Fig. 5, lower panel). The region of C23/nucleolin which bound phosphorothioate oligonucleotides in this assay contains the RNA recognition motifs (RRM) of nucleolin. The shortest C23/nucleolin fragments to which binding of phosphorothioate oligonucleotides was detected (286–539 and 423–707) contain three 11-amino acid RRMs (domains A–C and B–D, respectively); the fragments containing only RRM A or A and B (286–411 and 286–449, respectively) did not bind phosphorothioate oligonucleotides. Studies are currently in progress to determine precisely the minimal region required for oligonucleotide binding and the relative contribution of each RRM.

4. Discussion

Studies that explored the internalization and subcellular localization of oligonucleotides demonstrated that both phosphodiester and phosphorothioate oligonucleotides accumulate predominantly in the cell nucleus [8–13]. Previous in vitro binding of phosphorothioate oligonucleotides to nuclear proteins has been demonstrated [8,10,14], but specific proteins were not identified. The present study shows non sequence-specific in vitro binding of phosphorothioate oligonucleotides to nuclear proteins isolated from human LOX cells; very low level binding was found for cytoplasmic proteins. This binding was dependent on the presence of the phosphorothioate backbone. Only phosphorothioate oligonucleotides but not phosphodiester oligonucleotides competed with the binding of radiolabeled phosphorothioate oligonucleotides.

A 110 kDa protein which had consistently strong phosphorothioate oligonucleotide binding signal was shown by immunoprecipitation to be C23/nucleolin. Binding to nucleolin was also confirmed using recombinant glutathione S-transferase/nucleolin fusion proteins. Nucleolin is a predominantly nucleolar protein which is associated with nucleolar chromatin in interphase and with NOR (nucleolar organizing regions) during mitosis [25,26]; it has been suggested to play a role in preribosome assembly [27]. C23/nucleolin may also function as a transcriptional repressor [28], a nucleus to cytoplasm shuttle protein [29], and a nuclear matrix attachment region protein [30].

C23/nucleolin has been demonstrated to bind RNA, with its RNA recognition motifs [31] as well as single stranded DNA [32–34]. Using GST/nucleolin constructs, we have shown that three RNA recognition motifs appear to be necessary for phosphorothioate oligonucleotide binding activity. The glycine-

arginine-rich (GAR) region at the carboxy terminus of C23/nucleolin may facilitate its binding to phosphorothioate oligonucleotides (compare amino acids 286–707 and 286–627 in Fig. 5). This GAR region has been shown to be essential for efficient binding of C23/nucleolin to RNA [34,35].

Binding of phosphorothioate oligonucleotides to C23/nucleolin and other nuclear proteins may relate to their effects on cell function. A strong binding to a nucleolar protein could potentially be toxic to normal cells by affecting their growth and reproduction. For example, binding of phosphorothioate oligonucleotides to C23/nucleolin could inhibit its function in ribosomal RNA synthesis, resulting in severe toxicity. Such binding to nuclear proteins could be involved in some of the random effects noted for phosphorothioate oligonucleotides (reviewed in Stein and Cheng [5]). Studies on these effects need to be developed.

Several other phosphorothioate oligonucleotide binding proteins have been previously identified. An 80 kDa membrane protein isolated from CHO fibroblasts and HL-60 cells [6,7] was found to bind to both phosphorothioate and phosphodiester oligonucleotides. This binding was competed by other polyanions, including dextran sulfate and pentosan polysulfate. Other phosphorothioate and phosphodiester binding proteins are recombinant soluble CD4, the T-cell surface receptor for the human immunodeficiency virus-1 envelope glycoprotein gp120 [36], gp120 [24] and protein kinase C, beta-1 isoform [37]. Binding of phosphorothioate oligonucleotides to gp120 was demonstrated to occur at the positively charged v3 loop domain, and was specific for phosphorothioate oligonucleotides; no binding was detected for phosphodiester oligonucleotides [24]. This result was similar to our finding with C23/nucleolin and other nuclear proteins. However, the v3 loop domain interacted with sulfated polysaccharides. Our data showed that the sulfated polysaccharides dextran sulfate and pentosan polysulfate did not compete with the binding of phosphorothioate oligonucleotides to C23/nucleolin, which indicates a different type of interaction than by simple cationic-anionic binding.

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