Characterization of a Potent and Specific Class of Antisense Oligonucleotide Inhibitor of Human Protein Kinase C-α Expression*

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The use of antisense oligonucleotides to inhibit the expression of targeted mRNA sequences is becoming increasingly commonplace. Although effective, the most widely used oligonucleotide modification (phosphorothioate) has some limitations. In previous studies we have described a 20-mer phosphorothioate oligodeoxynucleotide inhibitor of human protein kinase C-α expression. In an effort to identify improved antisense inhibitors of protein kinase C expression, a series of 2′ modifications have been incorporated into the protein kinase C-α targeting oligonucleotide, and the effects on oligonucleotide biophysical characteristics and pharmacology evaluated. The incorporation of 2′-O-(2-methoxy)ethyl chemistry resulted in a number of significant improvements in oligonucleotide characteristics. These include an increase in hybridization affinity toward a complementary RNA (1.5° C per modification) and an increase in resistance toward both 3′-exonuclease and intracellular nucleases. These improvements resulted in a substantial increase in oligonucleotide potency (>20-fold after 72 h). The most active compound identified was used to examine the role played by protein kinase C-α in mediating the phorbol ester-induced changes in c-fos, c-jun, and junB expression in A549 lung epithelial cells. Depletion of protein kinase C-α protein expression by this oligonucleotide lead to a reduction in c-jun expression but not c-fos or junB. These results demonstrate that 2′-O-(2-methoxy)ethyl-modified antisense oligonucleotides are 1) effective inhibitors of protein kinase C-α expression, and 2) represent a class of antisense oligonucleotide which are much more effective inhibitors of gene expression than the widely used phosphorothioate antisense oligodeoxynucleotides.

The protein kinase C (PKC)1 family of isozymes is composed of at least 11 different, but structurally related serine/threonine kinases. These can be subdivided on the basis of structural and biochemical similarities into three groups, the conventional (α, β, βII, and γ), the novel (δ, ε, η, θ, and µ), and the atypical (ζ and ι) (1–3). The classic PKCs and the novel PKCs are activated by 1,2-diacylglycerol, which is generated by phospholipase cleavage of membrane phospholipids. These phospholipases are regulated by many growth factors and hormones, and it is therefore widely thought that PKC isozymes play an important role in regulating cell proliferation and differentiation, as well as short-term cellular responses, such as secretion and ion flux (1). The identification of multiple members of the PKC family has lead to speculation that individual isozymes play different roles in regulating different cell functions (4). Much evidence is available to support this hypothesis. For example, expression profiles of the individual family members is extremely heterogeneous, both at the tissue and the subcellular levels (4, 5). In addition, the substrate specificities of purified proteins are very different, and the responses of isozymes to stimuli differ not just between isotypes, but also between the same isotype stimulated in different cell types.

Considerable effort has been made over the last 10 years to develop isozyme-specific inhibitors of PKC to allow the dissection of the PKC-dependent signaling pathways (6). These efforts are hampered by the similarities in protein structure of the many isozymes of PKC, which make the identification of specific, small molecule enzyme inhibitors difficult. To overcome this difficulty, we have used antisense oligonucleotides to inhibit the expression of individual isozymes of PKC (7, 8). Antisense oligonucleotides can be targeted to mRNA sequences which are unique to a given PKC isozyme, leading to the selective inhibition in expression of that isozyme (9, 10). The long half-lives of some PKC proteins (and other proteins which have been targeted with antisense oligonucleotides) have proven problematic, as we have found that the most widely used oligonucleotide modification available, the phosphorothioate (P=S) oligodeoxynucleotide, is metabolized in cells over time. This leads to a loss of activity over a 48–72-h period, which can make inhibition of some PKC isozymes difficult (7, 8, 11).

The factors which govern oligonucleotide activity are complex. Two important parameters are the affinity with which an oligonucleotide hybridizes to a target mRNA, and the ability of the oligonucleotide to withstand degradation by intracellular nucleases (12–19). We have therefore sought to improve these characteristics, with the anticipation that this would lead to the identification of antisense oligonucleotides with improved pharmacological activity compared with those presently available. This should allow for the development not just of improved antisense inhibitors of PKC, but of a more generalized class of antisense effective against any mRNA target which encodes a protein with a long half-life.

In the present study, the biophysical and pharmacological activity of oligonucleotides containing the recently described 2′-O-(2-methoxy)ethyl (2′-MOE) modification (20), with both...
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2-O-methyl (2'-OM) and 2'-deoxy containing oligonucleotides are contrasted. Antisense oligonucleotides can inhibit the expression of proteins by a number of potential mechanisms (21-24). One of the most effective oligonucleotide-dependent mechanisms for reducing protein expression is to cause an RNase H-mediated cleavage in the hybridized target mRNA (7, 25, 26). Unfortunately, however, 2'-alkyl modifications (such as 2'-MOE) do not support RNase H-mediated mRNA cleavage (12, 18, 27, 28). This can be overcome by the inclusion of 2'-deoxy residues into an antisense oligonucleotide, in combination with 2'-alkyl modifications, in a motif that will support RNase H cleavage (chimeric oligonucleotides) (11, 15, 29-31). In our studies reported here, an oligonucleotide containing 2-MOE modification in such a configuration was found to be at least 20-fold more active than conventional P = S oligodeoxy nucleotides at reducing the expression of PKC-α mRNA in A549 lung carcinoma cells. This inhibition resulted in a time dependent and oligonucleotide-specific reduction in expression of PKC-α protein. This has allowed us to examine the role played by PKC-α in regulating the expression of members of the AP-1 family of transcription factors in A549 lung carcinoma cells. Activation of PKC by phorbol esters leads to an increase in expression of the fos and jun family members, by both increased transcription and increases in mRNA stability. The PKC isoform responsible for this increase is not clear, as A549 cells express multiple phorbol ester binding PKC isozymes, including PKC-α, δ, ε, and η. Depletion of PKC-α from A549 cells had a dramatic effect on the increase in c-jun mRNA expression, reducing the up-regulation to levels seen in control cells. In contrast, the phorbol ester dependent increase in c-fos and junB mRNA expression was not inhibited.

EXPERIMENTAL PROCEDURES

Cell Culture—Human A549 lung carcinoma cells were obtained from the American Type Tissue Collection (ATCC). Cells were grown in Dulbecco’s modified Eagle’s medium containing 1 g of glucose/liter and 10% fetal calf serum and routinely passaged when 90–95% confluent.

Oligonucleotide Synthesis—Phosphorothioate oligodeoxy nucleotides were synthesized as described previously (7). 2'-O-Methyl and 2'-MOE oligonucleotides were synthesized as described (32).

Measurement of Oligonucleotide Tₘ—Absorbance versus temperature profiles were performed as described previously (33). Briefly, antisense oligonucleotides were hybridized to complementary RNA strands and Tₘ values and free energies of duplex formation were obtained. Values are the averages of three experiments.

Oligonucleotide Treatments of Cells—A549 cells were grown to 60–70% confluence in T-75 flasks. The cells were then washed twice with Dulbecco’s modified Eagle’s medium and then 5 ml of Dulbecco’s modified Eagle’s medium containing 20 μg/ml N-[1-(2, 3-dioleyloxy)propyll]-n, n, n-trimethylammonium chloride/dioleyl phosphatidylethanolamine (DOTMA/DOPE) (Lipofectin®)(Life Technologies, Inc.) solution was added to the solutions. 5 ml of Dulbecco’s modified Eagle’s medium containing 20 μM stock solution and the flask swirled to mix the solutions. The cells were then incubated at 37°C for 4 h and then the DOTMA/DOPE/oligonucleotide solution was aspirated off and replaced with medium for the indicated time.

In Vitro Nuclease Stability—Oligonucleotide resistance to snake venom 3'-phosphodiesterase was determined as described previously (11). Briefly, the oligonucleotides were gel purified and 5'-end-labeled with high performance liquid chromatography-purified [γ-³²P]ATP (ICN). The oligonucleotides (100 nM) were then incubated with snake venom phosphodies-

terase (U. S. Biochemical Corp./Amersham) (5 × 10⁻³ units/ml) for the indicated times. The oligonucleotide metabolites were then resolved on a 20% denaturing polyacrylamide gel followed by quantitation by PhosphorImager (Molecular Dynamics) analysis.

Oligonucleotide Metabolism in A549 Cells—A549 cells were treated with 500 nM oligonucleotides as described above and allowed to recover for 72 h. At this time, metabolites were recovered and analyzed by capillary gel electrophoresis as described previously (11). After digestion with proteinase K, oligonucleotide metabolites were recovered by sequential passages through an anion exchange column and a reverse phase column. Analysis of the samples by capillary gel electrophoresis was performed on a Beckman 5010 PACE capillary electrophoresis unit.

Measurement of PKC, jun, and fos mRNA Expression—Total mRNA was extracted from cells and resolved on agarose gels as described previously (7). These were transferred to nylon membrane (Bio-Rad) and probed with [³²P]cDNA probes for c-fos, c-jun, junB (ATCC). Gels were then stripped and reprobed with radiolabeled human glyceral-3-phosphate dehydrogenase (GAPDH) probe to confirm equal loading. Radioactive bands were quantitated using a PhosphorImager, and typically we measure only the upper of the two PKC-α transcripts, although both are reduced with

![FIG. 1. Effect of number of oligodeoxy residues on the ability of a phosphorothioate oligodeoxy nucleotide to reduce PKC-α mRNA expression. A. A549 cells were treated with oligodeoxy nucleotides (500 nM) and DOTMA/DOPE as described under “Experimental Procedures” for a period of 4 h. Cells were then washed and allowed to recover for a further 20 h. At this time PKC-α mRNA expression was determined by Northern blotting. The gels were stripped and reprobed for expression of a housekeeping gene (G3PDH) to confirm equal loading. The oligodeoxy nucleotides used were all full P = S. Oligodeoxy gap size refers to the number of contiguous centrally placed 2'-oligodeoxy residues, with the remaining residues being 2'-O-methyl. B: quantitation of the above gel.](image)
identical kinetics upon oligonucleotide treatment of cells. 2

Measurement of PKC Protein Expression—PKC isozyme protein expression was determined by Western blotting (7). The antibodies used were obtained as indicated. PKC-α, UBI; PKC-δ, Santa Cruz Biotechnology; PKC-ε, a gift from Dr. Doriando Fabbro, Novartis Pharmaceuticals; PKC-η, BioMol; PKC-μ, Santa Cruz Biotechnology; and PKC-ζ (UBI).

Measurement of PKC Enzyme Activity—A549 cells were treated with oligonucleotides for 3 days. Cells were then washed in cold phosphate-buffered saline, scraped, and pelleted into a sample preparation buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine). A cytosolic fraction was prepared by centrifugation at 100,000 g for 1 h at 4° C. PKC enzyme activity was determined by measuring the ability of the cytosolic protein extract to phosphorylate a synthetic peptide substrate in the absence or presence of phosphatidylserine in an enzyme-linked immunosorbent-based assay according to the manufacturers instructions (MBL Co. Ltd., Nagoya, Japan). The final concentrations of the reaction mixture used were 25 mM Tris-HCl, pH 7.0, 3 mM MgCl₂, 0.1 mM ATP, 2 mM CaCl₂, 0.5 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, ±50 μg/ml phosphatidyserine. PKC activity is defined as phosphatidyserine-dependent kinase activity.

RESULTS

Determination of Minimum Oligodeoxynucleotide Residues Required for Oligonucleotide Activity—The inhibition of PKC-α mRNA expression by the uniform phosphorothioate oligodeoxynucleotide sequence used here is believed to be mediated by RNase H (7). The 2'-modifications examined in the present study do not form substrates for RNase H, and therefore need to be incorporated into the oligonucleotide in combination with oligodeoxynucleotide residues to effect this mechanism of mRNA degradation (18) (20, 34). The number of contiguous oligodeoxynucleotide residues required in an oligonucleotide to support RNase H cleavage of a hybridized RNA have been proposed to range from 3 to 8 (15, 31, 35–39). To determine the requirements for this oligonucleotide sequence, we have initially incorporated 4, 6, or 8 contiguous oligodeoxynucleotide

![FIG. 2. Digestion of oligonucleotides by snake venom phosphodiesterases. Oligonucleotides were incubated with snake venom phosphodiesterase for the indicated times as described under “Experimental Procedures.” The digested oligonucleotides were resolved on 20% polyacrylamide gels and full-length 20-mer quantitated using a PhosphorImager.](http://example.com/image)

TABLE I
Sequence, chemistry, and hybridizing affinity of the oligonucleotides used in the present study

| Oligonucleotide | Structure and Sequence | Tₘ (°C) | 2' Substituent |
|-----------------|-----------------------|--------|---------------|
| ISIS 3521       | G₃T₅C₃T₅C₅G₅C₅T₅G₅S₅G₅T₅G₅S₅A₅G₅T₅T₅C₅A | 52.1   | (deoxy)       |
| ISIS 11485      | G₅O₅T₅O₅C₅O₅G₅O₅T₅O₅G₅O₅T₅O₅G₅O₅T₅O₅C₅A | ND     |               |
| ISIS 5357       | G₃T₅C₅T₅C₅G₅C₅T₅G₅S₅G₅T₅G₅S₅A₅G₅T₅T₅C₅A | 61.9   | (methoxy)     |
| ISIS 8329       | G₅O₅T₅O₅C₅O₅G₅O₅T₅O₅G₅O₅T₅O₅G₅O₅T₅C₅A | 62.6   | (methoxyethyl) |
| ISIS 9606       | G₃T₅C₃T₅C₃G₃C₃T₃G₃T₃G₃S₃G₃T₃G₃S₃A₃G₃T₃T₃C₃A | 64.8   |               |
| ISIS 9605       | G₅O₅T₅O₅C₅O₅G₅O₅T₅O₅G₅O₅T₅O₅G₅O₅T₅C₅A | 69.6   | (methoxyethyl) |
residues (deoxy gap) into the center of a full phosphorothioated 2'-O-methyl oligonucleotide, and determined the ability of these oligonucleotides to reduce PKC-α mRNA expression in human lung A549 cells at 500 nM concentration. As shown previously, a fully 2'-O-methyl modified compound was unable to reduce PKC-α mRNA expression (Fig. 1, A and B). However, increasing the number of oligodeoxynucleotide residues (deoxy gap) present in the oligonucleotide resulted in a progressive increase in the ability of the sequence to reduce PKC-α mRNA expression. A contiguous stretch of 8 oligodeoxy residues gave a greater than 90% reduction in expression of PKC-α mRNA (Fig. 1, A and B).

**Design and Hybridization Thermodynamics of 2'-Modified Oligonucleotides**—A series of oligonucleotides were subsequently synthesized with 8 contiguous central oligodeoxynucleotide residues flanked with either 2'-O-methyl or 2'-MOE-modified sugar residues. The 3' end base of each oligonucleotide was left oligodeoxynucleotide for synthetic reasons. P=S backbone linkages were always retained in the central oligodeoxynucleotide sequence of the oligonucleotide to maintain resistance to endonucleases in this part of the molecule (15). The flanking sequences were also prepared with either phosphorothioate and phosphodiester backbones (Table I). The incorporation of a total of 11 2'-O-methyl residues increased oligonucleotide affinity toward a complementary mRNA from 52.1 to 61.9° C and 62.6° C as either P=S or P=O backbone linkages. The 2'-MOE incorporations gave a greater increase in Tm° to 64.8 and 69.6° C, respectively.

**Oligonucleotide Nuclease Resistance**—The nuclease resistance of the chimeric oligonucleotides shown in Table I was determined using a number of different strategies. An in vitro nuclease assay was used to examine the ability to withstand digestion by a snake venom phosphodiesterase (a 3'–exonuclease). Under conditions which resulted in 50% digestion of the full-length P=S oligodeoxynucleotide (ISIS 3521) (60 min incubation time with the nuclease), approximately 75% of a chi-
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Fig. 5. Dose response for the reduction in PKC-α mRNA expression in A549 cells by 2′-MOE modified oligonucleotides 72 h after oligonucleotide addition to cells. A549 cells were treated with the indicated concentration of oligonucleotide (0, 50, 100, or 200 nM) and DOTMA/DOPE as described under “Experimental Procedures” for a period of 4 h. Cells were then washed and allowed to recover for a further 68 h. At this time PKC-α mRNA expression was determined by Northern blotting and quantitated using a PhosphorImager. The oligonucleotides used were ISIS 9605 (○), ISIS 9606 (●), ISIS 3521 (▲), ISIS 129663 (scrambled ISIS 9605) (★), and ISIS 13009 (scrambled ISIS 9606) (▲).

Fig. 6. Specificity of oligonucleotide mediated reduction in PKC-α mRNA expression. A549 cells were treated with 100 nM ISIS 9606 and DOTMA/DOPE, or DOTMA/DOPE alone as described under “Experimental Procedures” for a period of 4 h. Cells were then washed and allowed to recover for a further 20 h. At this time PKC-α, β, ε, η, and ζ mRNA expression was determined by Northern blotting.

meric 2′-O-methyl/P=O compound (ISIS 8329) was degraded (Fig. 2). In contrast, only approximately 40% of the 2′-MOE/P=O oligonucleotide (ISIS 9605) was degraded, demonstrating that even combined with a P=O backbone, this latter modification provides greater nuclease resistance than that obtained by a P=S oligodeoxynucleotide substituent. When the two 2′-O-modified sugar residues were evaluated in the context of a P=S backbone they provided considerable enhancement of stability (Fig. 2). The 2′-MOE was superior, demonstrating no digestion for the duration of the experiment.

Experiments were also performed to evaluate the effects of incorporating the 2′-O-methyl and 2′-MOE modifications as P=S on oligonucleotide stability in tissue culture cells. A549 cells were treated with oligonucleotides (500 nM in the presence of cationic liposomes) and the oligonucleotide metabolites extracted from cells 72 h later and resolved by capillary gel electrophoresis. At this time, extensive metabolism of ISIS 3521 had occurred consistent with the successive removal of 3′-bases by 3′-exonucleases resulting in the appearance of n-1, -2 etc. metabolites (Fig. 3). Some metabolism of the 2′-O-methyl containing oligonucleotide (ISIS 5357) was also apparent (results not shown). In contrast, no metabolism of ISIS 9606 (the 2′-MOE modified oligonucleotide) was found (Fig. 3).

Effect of 2′-Modifications on Ability of Oligonucleotides to Reduce Expression of PKC-α mRNA Expression—Transfection of the P=S oligodeoxynucleotide (ISIS 3521) into A549 cells results in a concentration-dependent reduction in PKC-α mRNA expression after 24 h (7). The IC50 for this reduction was approximately 100 nM. In the context of a P=S backbone, the 2′-O-methyl (ISIS 5357) and 2′-MOE (ISIS 9606) containing oligonucleotides demonstrate approximately a 2- and 5-fold increase in potency, respectively (Fig. 4A and B) as a consequence of the enhanced hybridizing affinity of these two compounds. In the context of a P=O backbone, the 2′-O-methyl oligonucleotide (ISIS 8329) is inactive, even though this molecule has a substantially higher Tm than the parent P=S oligodeoxynucleotide (ISIS 3521). In contrast, the phosphodiester containing 2′-MOE compound (ISIS 9605) is about 4-fold more active than ISIS 3521 (Fig. 4, A and B).

PKC-α protein has a very long half-life (approximately 24 h) (40) and therefore to substantially reduce expression of this protein (by >80%) should require oligonucleotide activity for at least three half-lives of the protein. The ability of the 2′-MOE modification to withstand nuclease digestion prompted us to determine whether oligonucleotides containing these modifications could reduce expression of PKC-α mRNA for extended periods of time. Oligonucleotides ISIS 3521, ISIS 9605, and ISIS 9606 (as well as two scrambled control oligonucleotides) were transfected into A549 cells and PKC-α mRNA expression determined 72 h later. At this time ISIS 3521 is inactive at concentrations up to 100 nM, whereas ISIS 9605 and ISIS 9606 are able to maintain reduced levels of PKC-α expression with an oligonucleotide IC50 of approximately 100 nM (Fig. 5). The specificity in oligonucleotide-dependent reduction in PKC-α mRNA expression by 2′-MOE containing oligonucleotides was also examined. First, two scrambled control oligonucleotides
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DISCUSSION

Antisense oligonucleotides must possess certain characteristics in order for them to demonstrate full biological activity. Oligonucleotides must be sufficiently resistant to intracellular nucleases, have sufficient affinity for targeted mRNA to bind with a high degree of specificity and fidelity, and possess a

Fig. 7. Oligonucleotide mediated reduction in PKC-α protein expression in A549 cells. A, A549 cells were treated with 100 nM of either ISIS 9606 or ISSI 13009 and DOTMA/DOPE as described under "Experimental Procedures" for a period of 4 h. Cells were then washed and allowed to recover for either another 20, 44, or 68 h. At this time total cell protein was extracted and the expression of PKC-α, -δ, or -ζ determined by Western blotting. B, quantitation of the gels in A: PKC-α (△), -δ (∇), or -ζ (○).

Fig. 8. Oligonucleotide mediated reduction in PKC enzyme activity in A549 cells. A, A549 cells were treated with 100 nM of either ISIS 9606 or ISSI 13009 and DOTMA/DOPE as described under "Experimental Procedures" for a period of 4 h. Cells were then washed and allowed to recover for a further 68 h. PKC enzyme activity was determined by measuring the ability of a cytosolic protein extract to phosphatidylate a synthetic peptide substrate in the absence or presence of phosphatidylserine in an enzyme-linked immunosorbent-based assay. Solid bars are kinase activity in the presence of 50 μg/ml phosphatidylserine, open bars in the absence of phosphatidylserine. B, samples of protein extract used in the above assay were analyzed by Western blotting to confirm a specific reduction in PKC-α protein expression by ISIS 9606.

tein expression or kinase activity. The remaining kinase activity present after oligonucleotide is likely to be due to a combination of residual PKC-α protein as well as other phospholipid-dependent kinases present in A549 cells (PKC-δ, ε, and η).

Effect of PKC-α Reduction on Phorbol Ester-mediated AP-1 Gene Expression in A549 Cells—AP-1 is a sequence-specific transcriptional complex composed of members of the fos and jun families (41). Activation of the AP-1 family of transcription factors is complex, involving both transcriptional and post-translational regulation. The phorbol ester class of tumor promoters is known to be strong inducers of AP-1 via activation of PKC. In A549 cells, 12-O-tetradecanoylphorbol-13-acetate (TPA) causes a time-dependent accumulation of mRNA transcripts of multiple members of the AP-1, including c-jun, junB, and c-fos. A maximum increase in expression of these transcripts occurs at between 30 and 60 min after TPA addition (data not shown). In order to determine whether PKC-α is involved in regulating this response, A549 cells were treated with ISIS 9606 prior to treatment with TPA. The induction of both c-fos and junB mRNA were unaffected by PKC-α depletion, however, the up-regulation of c-jun was almost completely inhibited (Fig. 9). A scrambled control oligonucleotide was without effect on the up-regulation of any of these AP-1 family members (Fig. 9).

We next determined whether ISIS 9606 was able to deplete cells of PKC enzyme activity. Using an enzyme-linked immunosorbent-based assay which measures phosphorylatedenzyme-dependent kinase activity we were able to demonstrate an overall reduction in PKC enzyme activity of approximately 70% (Fig. 8A). Samples of protein extract used in the assay were analyzed by Western blotting to confirm a specific reduction in PKC-α protein expression by ISIS 9606 (Fig. 8B). A scrambled control oligonucleotide (ISIS 13009) was without effect on PKC-α protein expression and kinase activity. The ability of ISIS 9606 to maintain reduced levels of PKC-α mRNA expression for up to 72 h suggested to us that this oligonucleotide would effectively reduce PKC-α protein expression. A549 cells were treated with 100 nM ISIS 9606 and PKC-α protein expression was determined by Western blotting. PKC-α protein expression was reduced in a time-dependent manner, consistent with a half-life of the protein of approximately 24 h. The oligonucleotide-mediated reduction in PKC-α protein expression was again shown to be specific for this isozyme, as levels of PKC-δ and -ζ were unaffected by ISIS 9606 (Fig. 7).

Effect of 2′-MOE Modified Oligonucleotide on PKC-α Protein Expression and PKC Enzyme Activity in A549 Cells—The ability of ISIS 9606 to maintain reduced levels of PKC-α mRNA expression for up to 72 h suggested to us that this oligonucleotide would effectively reduce PKC-α protein expression. A549 cells were treated with 100 nM ISIS 9606 and PKC-α protein expression was determined by Western blotting. PKC-α protein expression was reduced in a time-dependent manner, consistent with a half-life of the protein of approximately 24 h. The oligonucleotide-mediated reduction in PKC-α protein expression was again shown to be specific for this isozyme, as levels of PKC-δ and -ζ were unaffected by ISIS 9606 (Fig. 7).

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mechanism for inhibiting the expression of the protein encoded in the target mRNA. The instability of conventional P=O DNA to nucleases largely precludes their use as antisense oligonucleotides. This has lead to the replacement by a sulfur atom for an equatorial oxygen atom in the phosphate backbone of oligodeoxynucleotides, resulting in the widely used P=S modification (17, 23, 42). The P=S modification provides considerable stability to both exo- and endonucleases, and are widely used as specific inhibitors of gene expression. The advantages in nuclease stability obtained with this modification come with a price, as each incorporation of a P=S generates a chiral center and reduces the binding affinity for target mRNA by 1–0.5°C. Furthermore, although P=S oligonucleotides are less sensitive to nucleases, they will degrade in cells over time (11).

To overcome these drawbacks, considerable research has been undertaken to identify oligonucleotide modifications which provide a more attractive pharmacological profile than P=S oligodeoxynucleotides (27, 43, 44). These include modifications to the oligonucleotide backbone (44–46), the oligonucleotide base (47, 48), and the C2′ position of the ribose (15, 49–52). To date, however, very few modifications have been fully characterized with respect to their pharmacological activity in tissue culture and animals. In the present study, we have characterized the biophysical and pharmacological activities of oligonucleotides containing 2′-MOE modifications and compared them to both P=S oligodeoxynucleotides and 2′-O-methyl containing oligonucleotides. As 2′-MOE and 2′-O-methyl modifications do not support RNase H-mediated cleavage of hybridized mRNA, we have generated “chimeric” oligonucleotides, composed of both 2′-modifications and 2′-deoxy residues.

The incorporation of 2′-MOE modifications into a 20-mer oligonucleotide has a dramatic effect on the ability of the sequence to hybridize to a target mRNA, approximately 1.5°C per base compared with approximately 1°C per base for the 2′-O-methyl modification. The increase in hybridizing affinity obtained by the 2′-MOE modification is thought to result from the conformation of the sugar and the backbone. The sugar pucker is believed to change from C2′-endo (associated with B-form DNA) to C2′-exo, which more closely resembles RNA, and RNA/RNA duplexes are more stable than DNA/DNA duplexes (20). In addition, this limited rotational freedom may produce an enhanced steric effect that limits nucleases from digesting the 3′-phosphodiester, resulting in the increased nuclease resistance seen with this modification. Irrespective of the mechanism involved, the 2′-MOE modification enhances the nuclease resistance of a P=O oligonucleotide to at least that of a P=S oligonucleotide. Combined with a P=S modification, the 2′-MOE containing oligonucleotide exhibits further resistance to intracellular nucleases.

Increases in oligonucleotide affinity for a targeted mRNA have been reported to result in increased oligonucleotide potency, provided a mechanism for preventing protein synthesis is maintained (15, 19, 28, 32, 48). The 2′-MOE modified oligonucleotides were about 5-fold more potent than the parent P=S oligodeoxynucleotide sequence (at 24 h) and >20-fold more potent after 72 h, both in the context of either a P=S or a P=O backbone. The increase in potency for the 2′-MOE modification versus the P=S oligodeoxynucleotide is the result of a combination of enhanced nuclease resistance an increase in hybridizing affinity. This modification is clearly superior to the 2′-O-methyl modification. The increased potency of the oligonucleotide did not lead to any decrease in specificity. Control oligonucleotides containing the same chemistry as the active sequence, but with a randomized base composition were without effect on PKC-α mRNA and protein expression. In

Fig. 9. Effect of PKC-α depletion on the phorbol ester-mediated increase in transcription factor mRNA expression. A, A549 cells were treated with 100 nM of either ISIS 9606 or ISSI 13009 and DOTMA/DOPE as described under “Experimental Procedures” for a period of 4 h. Cells were then washed and allowed to recover for a further 68 h. Cells were then treated with 100 nM TPA for 30 min and the expression of either c-fos, junB, c-jun, or GAPDH mRNA expression determined by Northern blotting. B, quantitation of the expression of c-fos, junB, and c-jun, mRNA shown in A.
addition, only expression of PKC-α was reduced by oligonucleotide treatment of the cells. Relatively little is known about the specific signaling roles played by individual members of the PKC family of isozymes, although recent observations are beginning to shed light on this field. For example, PKC-μ appears to associate with the B-cell antigen receptor complex and is involved in regulating lymphocyte signaling (53); PKC-γ is thought to mediate transcriptional activation of the human transglutaminase 1 gene (54); and PKC-θ has been shown to selectively stimulate the transcription factor complex AP-1 in T-lymphocytes (55). Our strategy has been to use oligonucleotides to help define functions for PKC isozymes, this has proven successful for PKC-α, -ε, and -ζ (7) (56, 57).

Our previous studies indicated that PKC-α expression was required for the phorbol ester-mediated up-regulation of the cell adhesion molecule ICAM-1 expression in A549 cells (7). The 5'-regulatory domain of the ICAM-1 gene has been shown to contain multiple promoter sites (58–60). Of these, the most important regulators of ICAM-1 gene expression appear to be NFkB and TPA-responsive elements (61). In A549 cells the key regulators of AP-1 that are expressed are c-jun, junB, and c-fos (Fig. 9).2 The effectiveness of the 2'-MOE modified oligonucleotides at reducing PKC-α protein expression allowed us to examine whether the phorbol ester-mediated increase in expression of these three transcription factors could be regulated by this PKC isozyme. Of the three, only the expression of c-jun was inhibited, suggesting that PKC-α increases ICAM-1 expression through a mechanism which requires c-jun expression.

In summary, we have described the pharmacological characterization of a potent and specific class of antisense oligonucleotide inhibitors of gene expression. These 2′-MOE containing oligonucleotides are at least 20 times more active than the widely used P-5′ oligodeoxynucleotides. This increase in activity is derived from increases in both hybridizing affinity toward the targeted mRNA and in a substantial increase in resistance toward intracellular nucleases. The oligonucleotide has been used to demonstrate a role for PKC-α in regulating c-jun expression in A549 epithelial cells, suggesting that this TPA-responsive element binding transcription factor may be required for complete up-regulation of ICAM-1 mRNA expression in response to phorbol esters.

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