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Selective Inhibition of A-Raf and C-Raf mRNA Expression by Antisense Oligodeoxynucleotides in Rat Vascular Smooth Muscle Cells: Role of A-Raf and C-Raf in Serum-Induced Proliferation

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SUMMARY

Raf kinases, cytoplasmic serine/threonine protein kinases, have been proposed as important participants in mitogen-induced signal transduction. However, the precise role that Raf kinase isozymes play in cellular responses such as proliferation has not been resolved. The present study investigates the ability of antisense phosphorothioate oligodeoxynucleotides (ODNs), targeted against rat C-Raf and A-Raf kinases, to reduce gene expression and proliferation of cultured rat A10 smooth muscle cells (SMCs). Exposure of A10 cells to ISIS 11061, an active C-Raf antisense ODN, resulted in a potent, dose-dependent inhibition (IC_{50} = 55 nM) of C-Raf mRNA and protein expression. This inhibition was completely dependent on ODN sequence because the incorporation of increasing numbers of mismatches (up to six) into the sequence resulted in sequential loss of potency. Similarly, a dose-dependent reduction (IC_{50} = 125 nM) in A-Raf gene expression was observed after treatment of cells with the active A-Raf ODN, ISIS 9069, whereas two scrambled controls were without effect. These results demonstrate that ISIS 11061 and ISIS 9069 reduced gene expression in a sequence-specific and isozyme-specific manner. Moreover, administration of ISIS 11061 and ISIS 9069 to rat SMCs resulted in a significant and potent diminution of serum-induced proliferation with corresponding IC_{50} values of 216 and 273 nM, respectively. Taken together, these results indicate that A-Raf and C-Raf kinases play an important role in regulating vascular SMC proliferation and that antisense-mediated inhibition of Raf kinase activity may serve as a therapeutic modality in the treatment of vascular proliferative disorders.

Raf kinases are cytoplasmic serine/threonine protein kinases that serve as central intermediates in a signal transduction pathway stimulated by growth factors, neurotransmitters, and oncogenes (1, 2). Typically, growth-promoting stimuli induce the GTP-bound form of Ras, which serves to anchor Raf to the plasma membrane (3, 4), where it becomes activated via an undefined phosphorylation mechanism that may involve protein kinase C and/or tyrosine protein kinases (5, 6). Phosphorylation of Raf initiates the sequential activation of a protein serine/threonine kinase cascade because Raf phosphorylates and activates MEK, which, in turn, phosphorylates MAP kinase. Once activated, MAP kinase phosphorylates numerous cytoplasmic proteins and nuclear transcription factors that are essential for cellular processes such as growth and differentiation (see Ref. 9 for review).

Currently, the mammalian Raf serine/threonine kinase family consists of A-Raf, B-Raf, and C-Raf, which share three domains termed CR1, CR2, and CR3 with various degrees of conservation (8, 9). Numerous studies have implicated the ubiquitously expressed C-Raf (10) in mitogen-induced signal transduction and cellular growth (8, 9, 11). For instance, inhibition of C-Raf function by antisense constructs, by oligonucleotides, or by the expression of kinase-deficient mutants results in refractoriness to growth factor-induced transformation (12), suppression of serum-induced transcription of growth-responsive genes (13), inhibition of serum- and phorbol ester-induced thymidine incorporation (14) and antitumor effects *in vivo* (15). The regulation of A-Raf and B-Raf, which are found predominantly in urogenital tissues and brain, respectively (10), has been less characterized. However, recent studies have shown that exposure of cultured cells to diverse mitogens, including serum, growth fac-

ABBREVIATIONS: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAP, mitogen-activated protein; FBS, fetal bovine serum; SMC, smooth muscle cell; DOTMA/DOPE, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride/dioleoylphosphatidylethanolamine; DMEM, Dulbecco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ODN, oligodeoxynucleotide.

tors, and phorbol ester, results in rapid phosphorylation of A-Raf and B-Raf, resulting in subsequent activation of MEK1 and MAP kinase (16–19). Despite this evidence, which implicates A-Raf, B-Raf, and C-Raf in MAP kinase activity induced by mitogenic agents, little is known regarding the role of these isozymes in cellular proliferation.

Excessive proliferation of vascular SMCs is a critical process underlying the development of atherosclerosis and restenosis after balloon angioplasty. Growth factors regulate proliferation by binding to receptors with intrinsic protein tyrosine kinase activity, resulting in the initiation of intracellular signaling cascades (20). To date, the precise molecular mechanisms that underlie excessive proliferation of SMCs remain poorly defined, although it is likely that the signals induced by diverse mitogens will converge at a common downstream effector. Inhibition of *in vitro* proliferation of vascular SMCs has been attained after down-regulation of c-*myc* (21), proliferating cell nuclear antigen (22), and the receptors for thrombin (23) and insulin-like growth factor 1 (24) by antisense ODNs. Moreover, ODNs directed against proliferating cell nuclear antigen or c-*myc* have inhibited neointimal proliferation of SMC *in vivo* in the rat carotid artery injury model (25, 26). Taken together, these studies demonstrate that antisense oligonucleotides are useful tools to assess the relative contributions of signal transduction components in proliferation and may serve as therapeutic agents in disease states associated with excessive SMC proliferation.

In the present study, we have identified phosphorothioate ODNs that potently and specifically inhibit the expression of either A-Raf or C-Raf in rat A10 cells. Furthermore, these ODNs have been used to delineate the role of these Raf isoforms in the proliferative response of rat SMC. Reduction in A-Raf and C-Raf expression results in a potent suppression of serum-stimulated cellular proliferation, suggesting that A-Raf and C-Raf play an important role in mediating the proliferative response in these cells.

Experimental Procedures

Materials. Rat aortic A10 SMCs were obtained from American Type Culture Collection (Rockville, MD). DOTMA/DOPE (lipofectin), DMEM, and FBS were purchased from GIBCO BRL (Gaithersburg, MD). Tissue culture flasks and 96-well plates were from Corning Glassworks (Corning, NY). The CellTiter Cell Proliferation Assay was purchased from Promega (Madison, WI). A monoclonal antibody against C-Raf was obtained from Transduction Laboratories (Lexington, KY).

Oligonucleotide synthesis. Synthesis and purification of phosphorothioate ODNs for tissue culture experiments were performed as described previously (15). The 20-mer RNA complement to ISIS 11061, UCC-UUG-GGG-GCA-GAA-UGC-UA, was synthesized as described previously (27).

Melting curves. Absorbance versus temperature curves were measured at 260 nm using a Response II spectrophotometer (Gilford, San Francisco, CA). The buffer contained 100 mM Na^+, 10 nM phosphate, and 0.1 mM EDTA, pH 7. ODN concentration was 4 μM each strand, determined from the absorbance at 85° and extinction coefficients calculated according to Puglisi and Tinoco (28). Thermal melting temperatures, free energies of duplex formation, and dissociation constants were obtained from fits of data to a two-state model with linear sloping base-lines (29). Reported parameters are averages of three experiments.

Cell culture. A10 rat aortic SMCs (passages 18–23) were grown in DMEM supplemented with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin at 37° in a 95% air/5% CO_2 humidified atmosphere. Cells were subcultured routinely when 90% confluent by aspiration of the growth medium followed by a 30-sec rinse with a solution of 0.5 mM EDTA/0.05% trypsin.

Treatment of cells with oligonucleotides. Cells were incubated with oligonucleotides at a concentration of 10–750 nM in DMEM supplemented with 0.2% FBS containing DOTMA/DOPE solution (lipofectin) at a concentration of 0.25 μg/10 nM oligonucleotide. After 4 hr, the medium was removed and replaced with DMEM containing 10% FBS.

Northern blot and Western blot analysis. For determination of mRNA levels by Northern blot analysis, total RNA was prepared from rat A10 cells by the guanidinium isothiocyanate procedure as detailed previously (15). Briefly, mRNA, purified and quantitated spectrophoretically, was loaded in equal amounts onto 1.2% agarose gels containing formaldehyde and transferred to nylon membranes. The blots were hybridized with a human C-Raf cDNA probe obtained from American Type Culture Collection or a A-Raf cDNA probe was prepared according to the following procedure. To obtain a cloned fragment of the A-Raf gene, two primers were designed from the published sequence of the human gene (GenBank accession no. X04790). The first primer (5′-CAG GCA GGC AAC TCA TCG G-3′) was used to generate a single-stranded cDNA from total RNA derived from A-549 cells (reverse transcriptase and protocol from Gibco BRL). This primer and a second primer (5′-CCG AGA TCT CAA GTC TAA CAA-3′) were then used in a polymerase chain reaction reaction (*Taq* polymerase and protocol from Perkin-Elmer Cetus, Norwalk, CT). The resultant fragment [473 base pairs (nos. 1475 through 1947 of X04790)] was cloned into pBluescript (Stratagene, Cambridge, UK) (plasmid no. rtb.877). The A-Raf and C-Raf cDNA probes did not cross-react to the different Raf isotypes. These probes were radiolabeled with [α-^{32}P]dCTP by random primer labeling using a commercially available kit (Promega) according to the manufacturer's instructions. Probes hybridized to mRNA transcripts were visualized and quantitated using a Molecular Dynamics Phosphorimager (Sunnyvale, CA), as described previously (30). Blots were routinely stripped of radioactivity by boiling and reprobed with a ^{32}P-radiolabeled G3PDH probe to confirm equal loading.

For determination of protein levels by Western blot, C-Raf protein levels were determined by immunoblotting with a C-Raf-specific monoclonal antibody as detailed previously (15). Proteins were visualized and quantitated by Phosphorimage analysis.

Measurement of proliferation. A10 cells, grown in 96-well microtiter plates (5000 cells/well) to 80% confluence, were washed twice with phosphate-buffered saline and growth-arrested by replacing the medium with DMEM containing 0.2% FBS for 24 hr. Cells were incubated with oligonucleotides as described above. The medium was removed and replaced with DMEM (without phenol red) containing 10% FBS in a final volume of 100 μl for 24 hr. Proliferation was measured using the CellTiter Cell Proliferation Assay, which measures the reduction, by living cells only, of the tetrazolium compound MTS to formazan. Briefly, 20 μl of MTS is added to each well during the last 3 hr of stimulation with mitogen and the plates are returned to the 37° incubator. The absorbance of formazan at 490 nm is recorded using a microplate reader (Molecular Devices, Menlo Park, CA). Data generated using the MTS conversion assay are identical to those generated by either [^{3}H]thymidine incorporation or direct cell counting (data not shown).

Results

Specific inhibition of C-Raf mRNA and protein expression in A10 cells after treatment with an active phosphorothioate C-Raf ODN. A series of 20-base phosphorothioate ODNs, designed to hybridize to various regions

on rat C-Raf or A-Raf mRNA, were synthesized and screened for their ability to inhibit C-Raf and A-Raf mRNA expression in rat A10 SMC. Cells were exposed to ODNs for 4 hr in serum-free media containing the cationic lipid DOTMA/DOPE, which has been shown previously to facilitate the cellular uptake and enhance the biological activity of phosphorothioate ODNs (31). After an additional 20 hr of incubation in the presence of 10% FBS, the effects on C-Raf mRNA were analyzed by Northern blot analysis. ISIS 11061 and ISIS 9069, which hybridize to the 3′ untranslated region of C-Raf and A-Raf mRNA (Table 1), respectively, were identified as the most potent ODNs that inhibited C-Raf and A-Raf gene expression (data not shown). The specificity of this antisense-mediated inhibition was addressed in subsequent experiments.

Treatment of A10 cells with increasing concentrations of ISIS 11061 resulted in a dose-dependent reduction in C-Raf mRNA levels with an IC_{50} value of 55 ± 20 nM (Fig. 1, A and B; Table 2). To test for sequence specificity, studies also were employed using a series of mismatched control ODNs that contain between 1 and 6 base mismatches incorporated into the sequence of ISIS 11061 (Table 1). The incorporation of sequential base mismatches into the sequence resulted in a significant reduction in the potency of these ODNs to inhibit and hybridize to C-Raf mRNA, indicating that the inhibition of C-Raf mRNA expression by ISIS 11061 is sequence-specific and supporting an antisense mechanism of action for this ODN (Fig. 1, A and B; Table 2). Furthermore, the levels of C-Raf protein, assessed by Western analysis, were markedly reduced after 48 hr of treatment with ISIS 11061 but not with ISIS 13492, a 3-base mismatch phosphorothioate control (Fig. 1C). Quantitative analysis revealed that C-Raf protein levels were 94% of untreated levels for ISIS 13492 (*lane 2*) and 12% of untreated levels for ISIS 11061 (*lane 3*). Lipofectin alone had no effect on C-Raf mRNA or protein expression (data not shown).

Specific inhibition of A-Raf gene expression in A10 cells after treatment with an active phosphorothioate A-Raf ODN. To examine the specificity of ODNs targeted against A-Raf gene expression, the effects of the active A-Raf ODN ISIS 9069 and scrambled controls were examined for their ability to alter mRNA levels. ISIS 9069 decreased the expression of A-Raf mRNA in rat SMC, 24 hr after treatment, in a concentration-dependent manner with an IC_{50} value of 125 ± 25 nM (Fig. 2, Table 2). In contrast, scrambled control ODNs, which have the same base composition as ISIS 9069

TABLE 1

Summary of synthesized antisense oligonucleotide sequences directed against rat A-Raf and C-Raf

Sequences are shown 5′ to 3′ and target the 3′-untranslated region of rat C-Raf and A-Raf cDNA.

ISIS	Sequence	mRNA target
11061	ATGCATTCTGCCCCCAAGGA	C-*Raf* active oligonucleotide
13490	ATGCATTCTCCCCCCAAGGA	1-base mismatch control
13491	ATGCATTCTCGCCCCAAGGA	2-base mismatch control
13492	ATGCATTCCCGCCCCAAGGA	3-base mismatch control
13493	ATGCATTCCCGTCCCAAGGA	4-base mismatch control
13494	ATGCATTACCGTCCCAAGGA	5-base mismatch control
13495	ATGCATTACCGTACCAAGGA	6-base mismatch control
9069	CTAAGGCACAAGGCGGGCTG	A-*Raf* active oligonucleotide
11898	CGGAACAGGTGTAGCGACCG	A-*Raf* scrambled control
11900	CTGAACAGGCGGAGCGACTG	A-*Raf* scrambled control

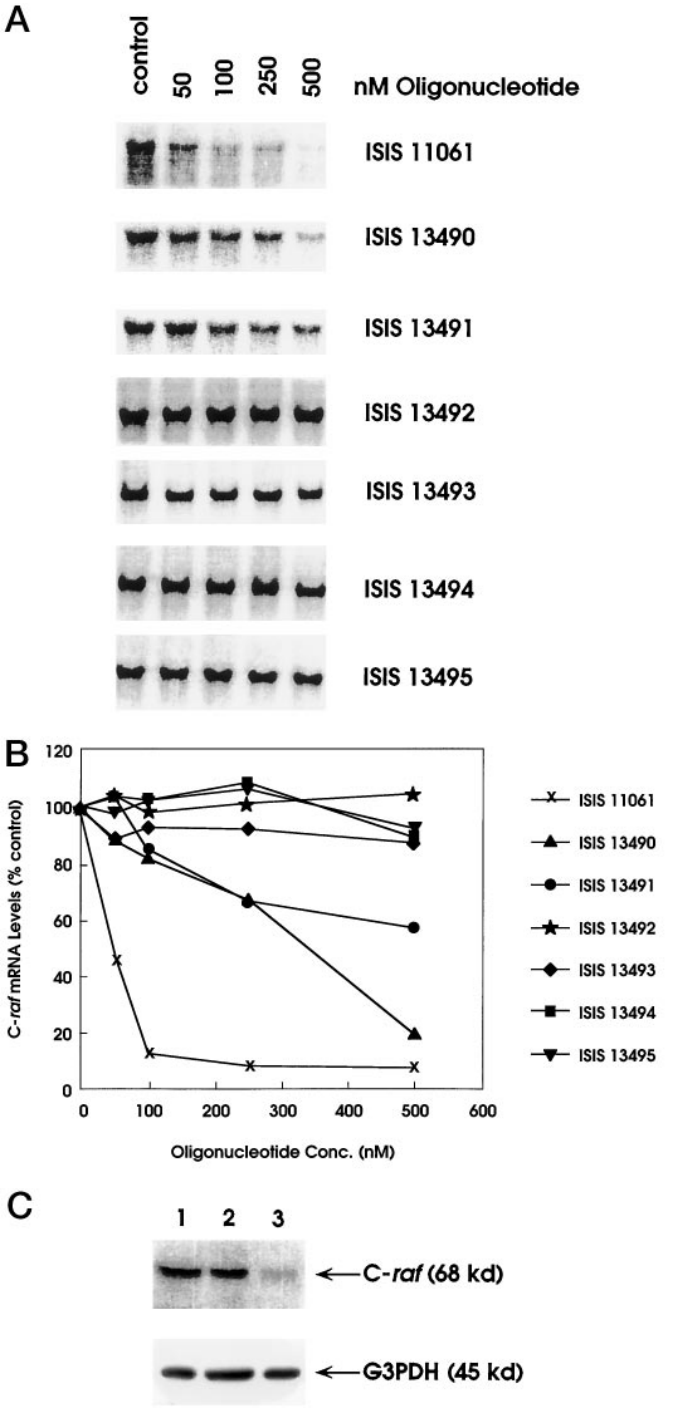

Fig. 1. Inhibition of C-Raf kinase mRNA and protein expression by ISIS 11061 and mismatched analogs in rat A10 cells. A, A10 cells were treated with increasing concentrations (50–500 nM) of the indicated antisense ODNs (see Table 1) and total RNA was prepared 24 hr later. Normalized RNA was analyzed for C-Raf mRNA levels by Northern blot analysis. "Control" indicates untreated cells. B, Quantitation of C-Raf mRNA levels from A. C-Raf mRNA levels were normalized to G3PDH mRNA levels (not shown) and quantitated by phosphorimage analysis as described under Experimental Procedures. The results are representative of three independent experiments. C, Western blot analysis of C-Raf kinase protein levels in A10 cells treated with ISIS 11061 (C-Raf specific) or ISIS 13492 (mismatched control ODN). Cells were treated with ODN at a concentration of 200 nM and protein extracts were prepared 48 hr later and C-Raf protein levels were determined as described under Experimental Procedures. *Lane 1*, no ODN treatment; *lane 2*, ISIS 13492 treatment; *lane 3*, ISIS 11061 treatment.

TABLE 2

Inhibition of mRNA expression and serum-induced proliferation induced by oligonucleotides directed against rat A-Raf and C-Raf in rat A10 smooth muscle cells

Rat A10 SMCs were treated with the indicated ODNs for 4 hr followed by replacement of media. After a 24-hr incubation, the effects of on mRNA expression and serum-induced proliferation were assessed as described under Experimental Procedures. Thermal melting temperatures (T_m) were determined for the indicated phosphorothioate ODNs targeted to ISIS 11061 complementary 20-mer RNA as described under Experimental Procedures. See Table 1 for ODN sequences. IC_{50} values were determined by visual inspection of the normalized graphs. Values shown are mean ± standard error for three to four experiments. N.D., not determined.

ISIS	T_m	IC_{50} value	
		mRNA expression	Proliferation
		nM	
C-Raf:			
11061 (active C-Raf ODN)	60.5	55 ± 20	216 ± 39
13490 (1 base mismatch control)	45.8	350 ± 45	251 ± 53
13491 (2 base mismatch control)	42.2	>500	240 ± 25
13492 (3 base mismatch control)	33.8	>500	408 ± 34
13493 (4 base mismatch control)	25.0	>500	>500
13494 (5 base mismatch control)	—	>500	>500
13495 (6 base mismatch control)	—	>500	>500
A-Raf:			
9069 (active A-Raf ODN)	N.D.	125 ± 25	273 ± 34
11898 (scrambled control)	N.D.	>500	>750
11900 (scrambled control)	N.D.	>500	>750

but contain sequences that are noncomplementary to A-Raf, were without effect, indicating that ISIS 9069 reduces A-Raf mRNA levels in a sequence-specific manner (Fig. 2; Table 1).

To further examine the specificity of antisense-mediated inhibition of gene expression, ISIS 11061 and ISIS 9069 were evaluated for their effects on C-Raf, A-Raf, and G3PDH mRNA levels. As shown in Fig. 3, exposure of A10 SMCs to 200 nM ISIS 11061 resulted in a complete and specific reduction of C-Raf mRNA while having no effect on A-Raf mRNA. Conversely, 200 nM ISIS 9069 completely inhibited A-Raf mRNA expression, whereas the expression of C-Raf kinase remained unchanged (Fig. 3). Therefore, ISIS 11061 and ISIS 9069 display isozyme-specific inhibition of C-Raf and A-Raf expression, respectively. Moreover, neither ODN effected G3PDH mRNA levels, demonstrating further selectivity for the targeted mRNA (Fig. 3).

Antiproliferative effects induced by ODNs directed against A-Raf and C-Raf in rat A10 cells. To examine whether reduction in A-Raf and C-Raf gene expression effected serum-induced proliferation, rat A10 SMCs were exposed to ODNs for 4 hr in the presence of DOTMA/DOPE and then stimulated for 24 hr with 10% serum. Under these experimental conditions, increasing concentrations of the active C-Raf ODN, ISIS 11061, significantly diminished serum-induced proliferation in a dose-dependent manner with an IC_{50} value of 216 ± 39 nM (Fig. 4A, Table 2). The sequential addition of mismatch bases into the ODN sequence of ISIS 11061 resulted in a gradual loss of the antiproliferative effect. ISIS 13490, a 1-base mismatch, and ISIS 13491, a 2-base mismatch, inhibited serum-induced proliferation with similar potency to ISIS 11061 (Fig. 4A, Table 2). Incorporation of three mismatches into ISIS 13492 resulted in a loss of potency and generated an IC_{50} value of 408 ± 34 nM (Fig. 4A, Table 2). In contrast, ISIS 13493, ISIS 13494, and ISIS 13495, which are 4-, 5-, and 6-base mismatches, were without

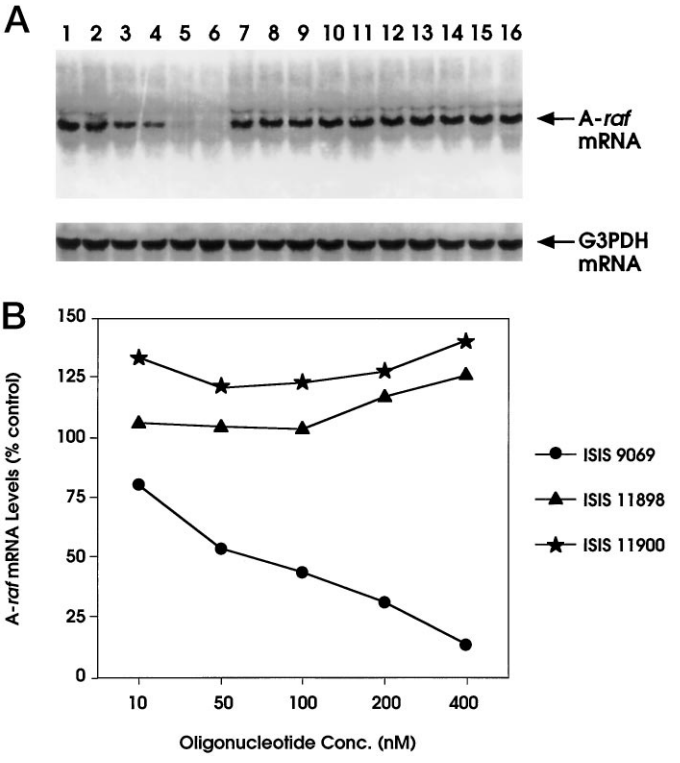

Fig. 2. Effect of ISIS 9069 and scrambled control ODNs on the expression of A-Raf kinase mRNA in rat A10 cells. A, A10 cells were treated with increasing concentrations of ISIS 9069 (A-Raf-specific), ISIS 11898 (scrambled control) or ISIS 11900 (scrambled control) (see Table 1) and total RNA was prepared 24 hr later. Normalized RNA was analyzed for A-Raf mRNA levels by northern blot analysis. *Lane 1*, no ODN treatment; *Lanes 2–6*, ISIS 9069 treatment at 10, 50, 100, 200 and 400 nM; *Lanes 7–11*, ISIS 11898 treatment at 10, 50, 100, 200 and 400 nM; *Lanes 12–16*, ISIS 11900 treatment at 10, 50, 100, 200 and 400 nM. B, Quantitation of A-Raf mRNA levels from Panel A. A-Raf mRNA levels were normalized to G3PDH mRNA levels (not shown) and quantitated by phosphorimage analysis as described under Experimental Procedures. The results are representative of three independent experiments.

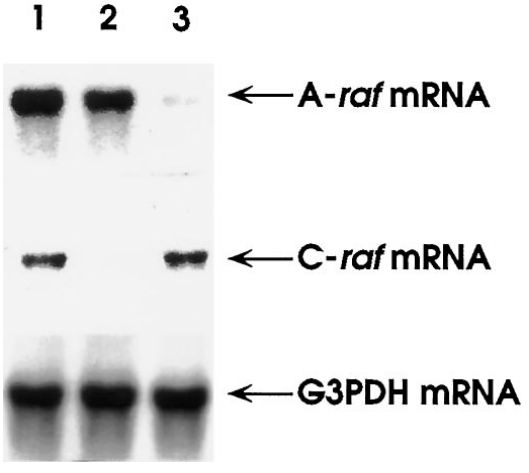

Fig. 3. Isozyme-specific inhibition of C-Raf and A-Raf mRNA expression in rat A10 SMCs treated with antisense ODNs. Northern blot analysis of C-Raf kinase, A-Raf kinase, and G3PDH mRNA levels in rat A10 cells treated with ISIS 9069 (A-Raf targeted, *lane 3*) or ISIS 11061 (C-Raf targeted, *lane 2*) or untreated (*lane 1*). ODNs were administered at a concentration of 200 nM and mRNA was prepared and analyzed 24 hr later, as described under Experimental Procedures.

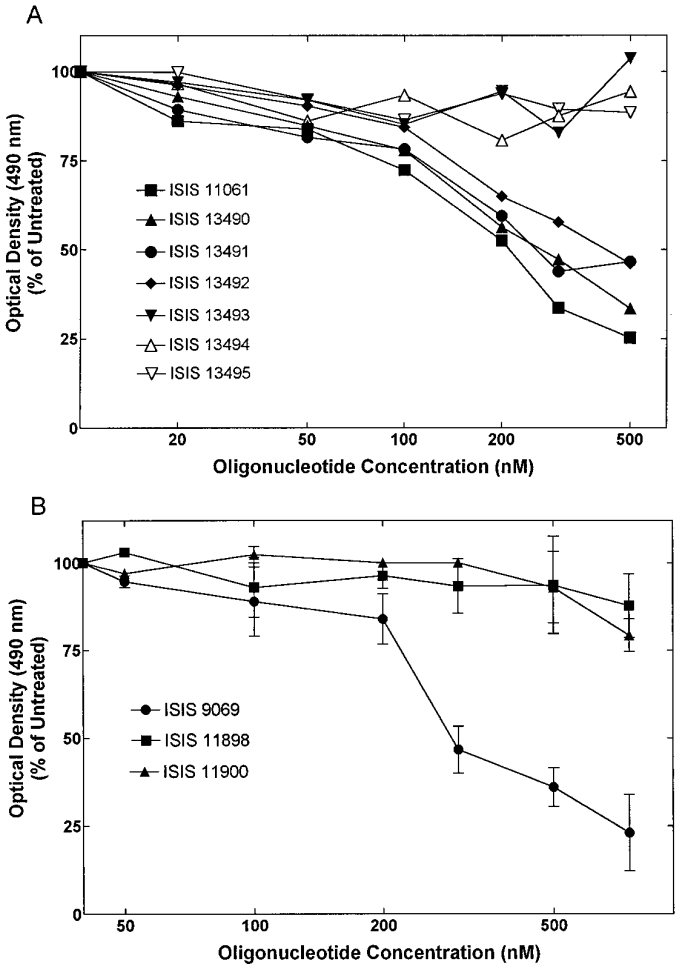

Fig. 4. Phosphorothioate oligonucleotides directed against A-Raf and C-Raf inhibit serum-induced proliferation in rat A10 SMC in a specific and dose-dependent manner. Growth-arrested cells were incubated with increasing concentrations of ISIS 11061 and mismatch controls (A) or ISIS 9069 and scrambled controls (B) in the presence of the cationic lipid DOTMA/DOPE. After 4 hr, the cells were treated with growth media containing 10% serum and proliferation was assessed 24 hr later using the MTS conversion assay. Values shown are the mean ± standard error obtained from three to four separate experiments and are expressed relative to cells that were not treated with ODNs. Error bars are not shown in A for the sake of clarity.

effect on serum-induced proliferation when tested at concentrations up to 500 nM (Fig. 4A, Table 2).

Pretreatment of rat vascular SMCs with increasing concentrations of ISIS 9069, the active phosphorothioate ODN directed against A-Raf, resulted in a significant diminution of serum-induced proliferation with an IC_{50} value of 273 ± 34 nM (Fig. 4B, Table 2). In contrast, ISIS 11898 and ISIS 11900, scrambled control ODNs, were without effect (Fig. 4B, Table 2). The proliferative response to serum was not altered in cells treated with lipofectin alone or with oligonucleotides, at concentrations up to 750 nM, in the absence of cationic lipid (data not shown).

To assess whether inhibition of SMC proliferation could be further augmented after reduction in A-Raf and C-Raf gene expression, rat A10 cells were exposed to ISIS 9069 and ISIS 11061 concurrently for 4 hr in the presence of DOTMA/DOPE and then stimulated for 24 hr with 10% serum. As shown in Fig. 5, treatment of SMC with increasing concentrations

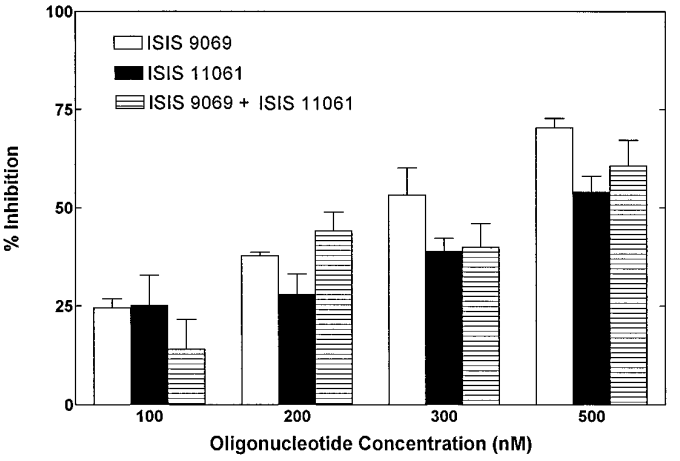

Fig. 5. Inhibition of serum-induced cell proliferation by ISIS 9069 and ISIS 11061 is not additive. Growth-arrested SMC were incubated with the indicated concentrations of ISIS 9069, ISIS 11061, or a combination of both oligonucleotides for 4 hr in the presence of the cationic lipid DOTMA/DOPE. Cells were then stimulated with 10% serum and proliferation was assessed by MTS conversion assay after 24 hr. Data are expressed relative to cells that were not treated with ODNs and are the mean ± standard error of triplicate determinations from one of three similar experiments.

(100–500 nM) of ISIS 11061 or ISIS 9069 resulted in a similar reduction of serum-stimulated proliferation. Administration of both ODNs together did not result in further attenuation relative to that observed in the presence of either ODN administered alone.

Discussion

In the present study, ISIS 11061 and ISIS 9069, 20-base phosphorothioate ODNs, were found to potently inhibit the expression of A-Raf or C-Raf mRNA as well as serum-induced proliferation in rat A10 SMC in a manner that was both target-selective and sequence-specific. ISIS 11061 and ISIS 9069 dose-dependently reduced mRNA expression of either C-Raf or A-Raf (IC_{50} values of 55–125 nM), respectively, while having no effect on the expression of the alternate Raf isozyme or G3PDH. Moreover, scrambled controls of ISIS 9069 had no effect on A-Raf mRNA expression or on serum-induced proliferation, indicating a sequence-specific mode of action. In addition, incorporation of base mismatches into the sequence of ISIS 11061 resulted in a sequential loss of potency to inhibit the expression of C-Raf mRNA and protein as well as cellular proliferation. The ability of mismatched ODNs to inhibit C-Raf mRNA expression and proliferation correlated well with the exception of the 2-base and 3-base mismatch controls. Although the explanation for this discrepancy remains uncertain, it may be due to differences in cell densities employed in these two assays. Specifically, A10 SMCs are grown and treated with ODNs in 96-well plates at a lower density than cells grown in 75-cm^2 culture flasks for the mRNA analysis and, consequentially, would incorporate mismatch ODNs in a more efficient manner. Under these circumstances, when making comparisons between these two assays, the rank order of potency for the mismatch series would be the most significant parameter. The rank order of potency for the mismatch ODN series to inhibit C-Raf mRNA expression and serum-induced proliferation was identical, indicating an antisense mechanism although additional non-

specific effects cannot be excluded at present. It should be noted that phosphorothioate ODNs have been found, in some instances, to exhibit nonantisense-mediated effects (see Refs. 32–34 for reviews), although most of these nonspecific effects are not observed at low ODN concentrations (33) such as those employed in the present study. Recently, Monia *et al.* (15) reported that ISIS 5132, a 20-base phosphorothioate ODN targeted against human C-Raf, exhibited antitumor activity, which also was selective for target and ODN sequence, in a mouse tumor xenograft model. Taken together, these findings strongly suggest that the observed effects of ISIS 11061 and ISIS 9069 on C-Raf and A-Raf mRNA expression and proliferation in rat A10 SMC are highly specific and consistent with an antisense mode of action. The inhibition of mRNA by the phosphorothioate ODNs described in this report is most likely due to RNase H-mediated cleavage of RNA because the 2′-*O*-methylphosphorothioate analog of ISIS 11061, which is RNase H-resistant (35), is a less potent inhibitor of proliferation.[1]

Raf serine/threonine kinases have been identified as key constituents of the Ras-MAP kinase signaling cascade, a major pathway that serves to transduce mitogenic signals at the cell surface into nuclear transcription events (7, 8). A-Raf, B-Raf, and C-Raf kinases are related Raf family members that have differential tissue distribution (10) and are regulated by diverse mitogenic stimuli (1, 2, 17). Raf kinase isozymes serve as downstream effectors of Ras-GTP (9, 36, 37) and, as a consequence, serve as participants in *Ras*-dependent hyperproliferative disorders. It has been shown previously that the mitogenic induction of cell proliferation in rat A10 SMC is dependent on Ras (38) and that inhibition of cellular Ras after the expression of a dominant negative mutant reduces *in vivo* SMC proliferation after balloon injury to the rat carotid artery (39).

In this study, we have used an antisense approach to inhibit the expression of rat A-Raf and C-Raf mRNA to delineate the contribution of these Raf isozymes to serum-induced proliferation. To our knowledge, these results are the first to demonstrate that ablation of A-Raf or C-Raf gene expression in SMC results in a significant reduction of serum-induced proliferation, suggesting that A-Raf and C-Raf serve as important signal transduction components of this biological response. These results are consistent with several recent observations that have demonstrated a regulatory role of C-Raf kinase in the proliferation of cultured fibroblasts or tumor cell lines (12–15). Although the role that A-Raf kinase plays in cellular proliferation has not been investigated previously, recent reports have shown that this Raf isozyme is activated after mitogenic stimulation with serum and growth factors (17, 19). It was interesting to note that concurrent administration of the active A-Raf and C-Raf ODNs to rat SMC did not result in a further reduction of the ODN-induced antiproliferative effect. These data suggest that antisense inhibition of A-Raf or C-Raf kinases, either alone or in combination, results in a similar inhibition of serum-induced proliferation in these cells.

The ability to selectively target Raf kinase isozymes to inhibit vascular SMC proliferation suggests that these antisense compounds could serve to increase the current understanding of vascular disease as well as have therapeutic utility to suppress *in vivo* SMC division in hyperproliferative disorders such as restenosis after angioplasty. Previously, antisense ODNs directed against proliferating cell nuclear antigen, c-*myc* or c-*myb*, reduced neointimal formation in the carotid artery injury model of restenosis in rat and pig (25, 26, 40). The suppression of neointimal SMC proliferation was found to be specific for ODN sequence and dependent on the time course of target gene expression and kinetics of ODN delivery (26, 40). The importance of demonstrating a specific antisense mode of action, through the inclusion of proper ODN controls and analysis of target mRNA and protein expression, is imperative due to issues regarding the specificity of the antisense approach (see Refs. 32–34 for reviews). However, the ability of the human C-Raf-targeted ODN, ISIS 5132, to inhibit tumor cell proliferation in culture and *in vivo* in a manner consistent with an specific antisense mode of action (15) suggests that antisense-mediated inhibition of Raf kinase isozymes also would be effective against SMC proliferation *in vivo*.

In conclusion, the present findings demonstrate that treatment of rat A10 SMC with phosphorothioate ODNs directed against A-Raf or C-Raf mRNA results in a potent and specific degradation of mRNA and protein. In addition, ablation of these Raf isozymes by antisense ODNs significantly reduces serum-induced proliferation, suggesting that both A-Raf and C-Raf are important, if not crucial, intermediary signal transduction components for the proliferation of vascular SMC growth *in vitro*. These results suggest that Raf kinases are good pharmacological targets for the treatment of SMC-related diseases and that an oligonucleotide-mediated approach may serve as a useful therapeutic modality to prevent hyperproliferative disease states such as restenosis after angioplasty.

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