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Phosphorothioate-Capped Antisense Oligonucleotides to Ras GAP Inhibit Cell Proliferation and Trigger Apoptosis but Fail to Downregulate GAP Gene Expression

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We have studied the effects of an antisense oligonucleotide to Ras GAP in leukaemia cell lines. When terminal phosphorothioate linkages were introduced into this oligonucleotide, it caused major growth inhibition and apoptosis in the chronic myeloid leukaemia (CML) cell line K562, but had little effect on the promyelocytic leukaemia cell line HL60. Neither the expression of Ras GAP mRNA nor p120 GAP protein was downregulated by the antisense oligonucleotide, suggesting a non-antisense mechanism for growth inhibition. The antisense oligonucleotide contained GGC triplets which have previously been reported to inhibit the activity of $p210^{bcr\text{-}abl}$ both *in vitro* and *in vivo.* However, cellular phosphotyrosine levels were found to be unaffected, suggesting that the activity of $p210^{bcr\text{-}abl}$ was normal and that the antisense oligonucleotide may be interacting aptamerically with a different cellular protein. Since K562 is very resistant to apoptotic cell death, the identity of the putative target molecule would be of considerable interest.

Many growth factors which induce cellular proliferation are known to cause activation of $p21^{ras}$ proteins, leading to signalling via a number of potential effector pathways (1). In haematopoietic cells $p21^{ras}$ is activated following stimulation of cells with a variety of cytokines (2). However in these cells this may be more important for the inhibition of apoptosis rather than induction of proliferation (3). Active $p21^{ras}$ is also required for the survival of the growth factor independent chronic myeloid leukaemia (CML) cell line K562 (4). This cell line contains the *bcr-abl* oncogene, which encodes a protein tyrosine kinase known to cause activation of $p21^{ras}$, and expression of *bcr-abl* in K562 cells maintains resistance to apoptotic cell death (5,6).

The Ras GTPase activating protein (p120 GAP or GAP) causes inactivation of $p21^{ras}$ by catalysing the conversion of the active GTP-bound form to the inactive GDP-bound form (7). There is evidence suggesting that GAP may also function as an effector molecule, involved in downstream signalling (8,9,10).

In order to assess the function of GAP in haematopoietic cells, Skorski *et al* treated cells with antisense oligonucleotides and reported specific downregulation of GAP mRNA levels (11). This correlated with growth inhibition of primary CML cells and a CML cell line, BV173, but the growth of the promyelocytic leukaemia cell line HL60 was not affected.

Antisense oligonucleotides have been reported to induce cellular effects not related to the antisense mediated gene downregulation, but still requiring sequence specificity. In some cases these oligonucleotides have been shown to mediate aptameric interactions with specific proteins (12,13). In order to improve the stability of oligonucleotides the backbone chemistry can be changed from phosphodiester to phosphorothioate, since the latter is more resistant to nucleases. Alternatively, the termini of an oligonucleotide can be capped with phosphorothioate linkages, thus giving resistance specifically to exonuclease attack (14).

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A problem with phosphorothioate oligonucleotides is that they are particularly liable to induce non-antisense effects due to interactions with cellular proteins (15). Bergan *et al* noted a correlation between aptameric inhibition of $p210^{bcr\text{-}abl}$ kinase activity *in vitro* and GGC base triplets present in oligonucleotides containing phosphorothioate linkages (13). When K562 cells were treated with the phosphorothioate-capped version of this oligonucleotide, the inhibition of the endogenous $p210^{bcr\text{-}abl}$ could be detected as a decrease in total cellular phosphotyrosine (16).

To clarify the function of GAP in haematopoietic cells, we have studied the effects of both unmodified and phosphorothioate-capped antisense GAP oligonucleotides on the cell lines K562 and HL60. Interestingly, the phosphorothioate-capped antisense oligonucleotide was found to inhibit cell growth and induce apoptosis in K562 cells independently of antisense mediated gene downregulation, or aptameric inhibition of $p210^{bcr\text{-}abl}$ kinase.

MATERIALS AND METHODS

Cell culture. Cell lines were grown in QBSF-51 medium (Sigma Chemical Co, Poole, UK) with 1% fetal bovine serum (heat inactivated at 65°C for 1 hr), 100 U/ml penicillin and 100 μg/ml streptomycin.

Oligonucleotides. These were supplied by Genosys Biotechnologies Inc. (The Woodlands, TX, USA) ready purified for antisense work by desalting, HPLC and gel filtration. They were further purified by two rounds of ethanol precipitation with three final washes with 75% ethanol. The sequences were 5′-GGC CTC GGC CGC CAT CAT-3′ (antisense) and 5′-ATG ATG GCG GCC GAG GCC-3′ (sense), corresponding to the first 18 nucleotides of the human Ras GAP cDNA beginning with the initiation codon. The modified oligonucleotides were prepared with a phosphorothioate linkage between the first two and last two nucleotides.

RT-PCR for GAP mRNA. Total cellular RNA (0.5 μg) was reverse transcribed with 200 U Superscript II Moloney Murine Leukaemia Virus reverse transcriptase (Gibco BRL, Paisley, UK) and 100 pmol random hexamers in a total volume of 20 μl at room temp for 10 min followed by 42°C for 1 hr. The cDNA was amplified by PCR with the following primer sequences: 5′-TAA GAG AGA GTG ATC GGA GG-3′ (sense) and 5′-TGG CTC TGG TGG TGC AAC TG-3′. Amplification was kept in the exponential phase to allow quantitative comparison of band intensity (data not shown). Products corresponding to the expected size of 214 bp were visualised on ethidium bromide stained agarose gels.

Western blotting. Cells were lysed at 4°C for 1 hr with 50mM Tris HCL pH8.0, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 1 μg/ml Aprotinin, 1mM sodium pyrophosphate, 0.1mM sodium orthovanadate, 200mM sodium fluoride and 75 μg/ml PMSF and cleared by centrifugation at 12,000g for 10 min. The total protein concentration was determined by the BCA method (Pierce, Rockford, IL, USA) and lysates were resolved on 7% SDS-PAGE and transferred to a PVDF membrane (Amersham, Bucks, UK). The membrane was blocked with wash buffer (10mM Tris HCL pH7.5, 100mM NaCL, 0.1% Tween 20) + 1% BSA for 1 hr at room temp, incubated in the same buffer with monoclonal antibodies to GAP or phosphotyrosine (Transduction Labs, Lexington, KY, USA) for 1 hr, washed for 30 min, incubated with peroxidase-conjugated goat anti-mouse antibody in wash buffer + 5% skimmed milk for 1 hr, washed for 30 min and ECL detection (Amersham, UK) was performed.

RESULTS

Antisense GAP Oligonucleotides Do Not Cause Downregulation of GAP mRNA Levels in HL60 Cells

Antisense and sense phosphodiester oligonucleotides to GAP were added to cultures of HL60 cells and after 72 hr, RNA was extracted from the cells and semi-quantitative RT-PCR was performed to detect GAP mRNA. This showed that GAP mRNA levels were not downregulated by the antisense oligonucleotide (Fig 1A). When the dosage of oligonucleotide was doubled, and the cells were grown in a serum-free medium to improve the oligonucleotide half-life, GAP mRNA levels were still not decreased (data not shown). One possible explanation for the lack of gene downregulation was the short half life usually associated with unmodified oligonucleotides. Therefore oligonucleotides of the same nucleotide sequences but containing terminal phosphorothioate linkages were tried, but were found to not cause downregulation of GAP mRNA levels after 72 hr (data not shown). Neither unmodified or phosphorothioate-capped antisense GAP oligonucleotides were found to significantly affect the growth of HL60 cells (data not shown).

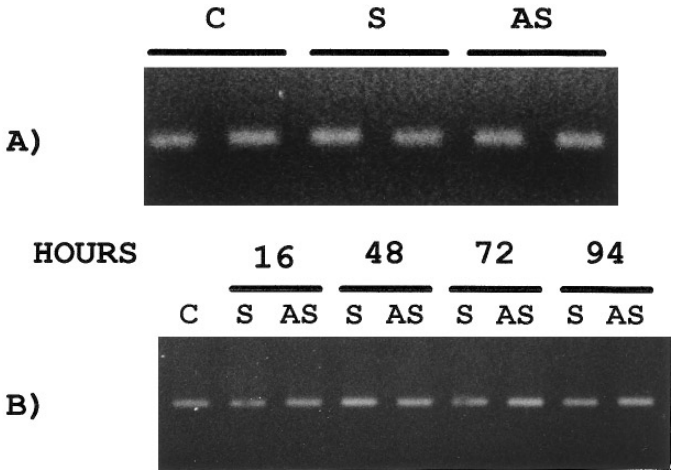

FIG. 1. RT-PCR for GAP mRNA. A) HL60 cultures were treated with 30μM sense (S) or antisense (AS) phosphodiester oligonucleotides with further doses of 15μM after 18hr and 40hr, or received no oligonucleotides (C), and after three days RNA was extracted, reverse transcribed and duplicate semi-quantitative PCR reactions were performed. B) K562 cultures were treated with 50μM sense (S) or antisense (AS) oligonucleotides containing terminal phosphorothioate linkages, with a second dose of 25μM after 40hr, or were untreated (C), and at the indicated timepoints RNA was extracted, reverse transcribed and semi-quantitative PCR reactions were performed.

Phosphorothioate-Capped Antisense GAP Oligonucleotides Inhibit the Growth of K562 Cells without Downregulating the Expression of GAP

Oligonucleotides with or without terminal phosphorothioate linkages were tested on cultures of K562 cells and it was found that the phosphorothioate-capped antisense oligonucleotide reproducibly caused strong growth inhibition (Fig 2). In contrast, cultures treated with either

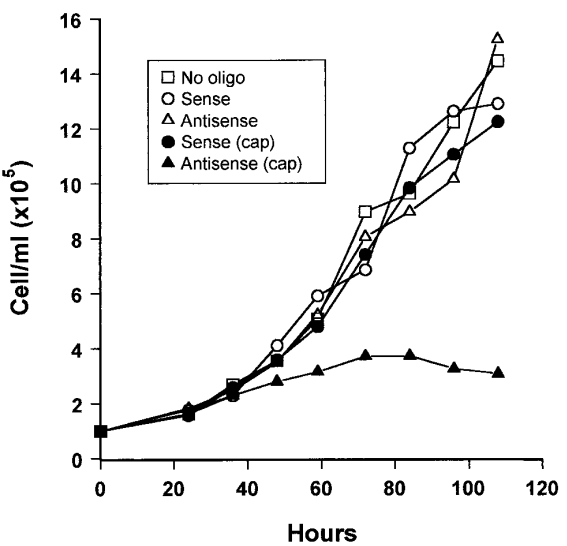

FIG. 2. Growth inhibition of K562 cells by antisense oligonucleotide. Cultures of K562 cells were treated with 50μM sense or antisense oligonucleotides with or without terminal phosphorothioate linkages (cap), and a second dose of 25μM after 40hr, or received no oligonucleotides, and viable cells were counted by trypan blue exclusion with a haemacytometer.

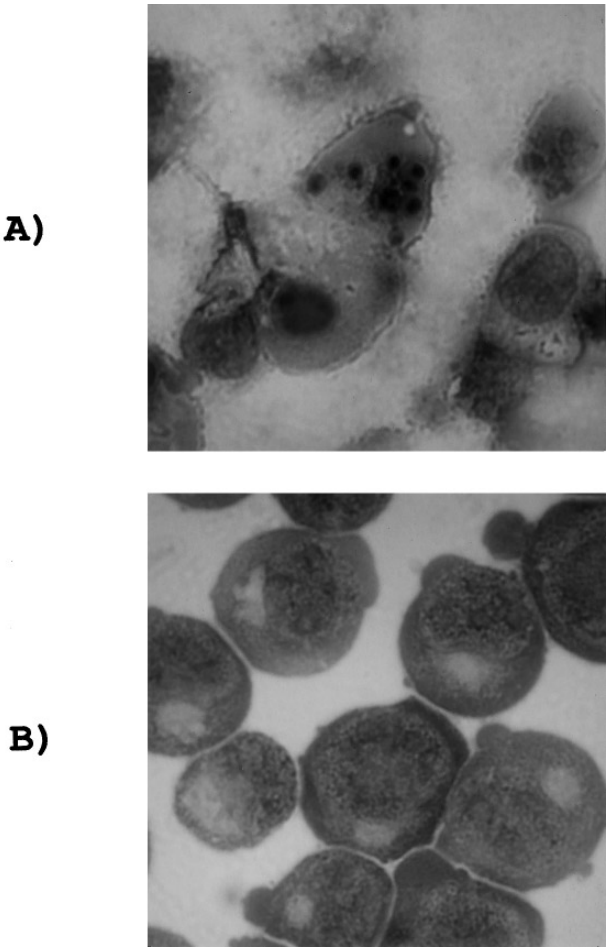

FIG. 3. Apoptosis of K562 cells treated with antisense oligonucleotide. Cultures of K562 were treated with 50μM antisense (A) or sense (B) oligonucleotides containing terminal phosphorothioate linkages, and after 65hr cells were spun onto microscope slides, fixed with methanol and stained with May-Grunwald-Giemsa.

the capped sense oligonucleotide or the unmodified antisense oligonucleotide grew at a similar rate to the untreated culture. Cytospin preparations from the culture treated with capped antisense oligonucleotide were stained with May-Grunwald-Giemsa and showed classic signs of apoptotic cell death, namely membrane blebbing, chromatin condensation and nuclear fragmentation (Fig 3A). Cells of the capped sense oligonucleotide treated culture resembled untreated cells in morphology (Fig 3B). To see if the antisense induced growth inhibition correlated with downregulation of GAP expression, RNA was extracted at four different time points and RT-PCR was performed. Surprisingly, GAP mRNA levels were found to be unaffected by the capped antisense oligonucleotide at all time points (Fig 1B). Similarly, Western blotting showed that GAP protein levels were also unaffected by antisense treatment over several time points (Fig 4A). Therefore it is clear that the antisense induced growth inhibition and apoptosis was not due to downregulation of GAP expression.

Cellular Phospho-tyrosine Levels Are Not Affected by the Antisense Oligonucleotide

It was noticed that the antisense but not the sense oligonucleotide contains two GGC base triplets. Since $p210^{bcr\text{-}abl}$ is known to confer K562 cells resistance to apoptotic cell death, and

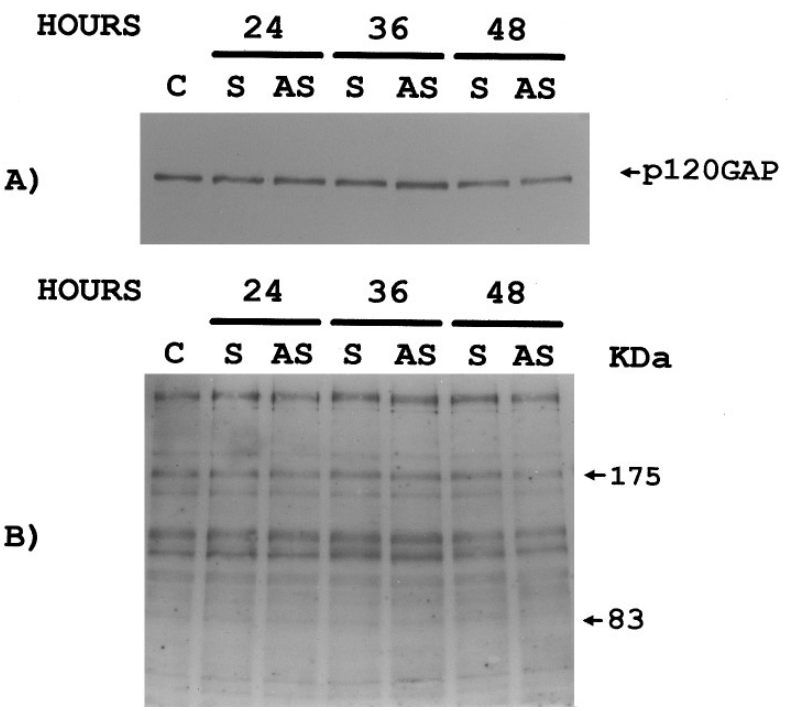

FIG. 4. Western blotting for GAP and phosphotyrosine. Cultures of K562 cells were treated with 50μM sense (S) or antisense (AS) oligonucleotides containing terminal phosphorothioate linkages and at the indicated timepoints cell lysates were prepared and subjected to SDS-PAGE and Western blotting with anti-GAP antibody (A) or anti-phosphotyrosine antibody (B).

phosphorothioate-capped oligonucleotides containing GGC triplets have been reported to inhibit $p210^{bcr\text{-}abl}$ kinase activity, one possible explanation for the antisense induced growth inhibition was inhibition of $p210^{bcr\text{-}abl}$. To investigate this, extracts of K562 cells treated with oligonucleotides were Western blotted with anti-phosphotyrosine antibodies. It was found that the capped antisense oligonucleotide did not cause a reduction in total cellular phosphotyrosine levels over several time points, indicating that $p210^{bcr\text{-}abl}$ tyrosine kinase activity was not inhibited (Fig 4B).

DISCUSSION

Antisense oligonucleotides provide a powerful means of studying the role of specific gene products in different cell types. We have observed growth inhibition and cell death induced by a phosphorothioate-capped antisense oligonucleotide to Ras GAP in a CML cell line K562, but only minor effects on the promyelocytic line HL60.

A serious shortcoming of the use of antisense oligonucleotides and especially ones containing phosphorothioate linkages, is the propensity to induce cellular effects not related to specific gene downregulation (15). This can be due to aptameric interactions with cellular proteins or may result from the degradation products, nucleotides and nucleosides which are known to affect cell growth (17). Our results indicate that the phosphorothioate-capped antisense GAP oligonucleotide inhibits the growth of the CML cell line K562, without affecting the levels of GAP mRNA or protein. In support of the observation that the effect was not due to downregulation of GAP expression, a stably transfected clone of K562 has been raised con-

taining an antisense GAP expression vector, which downregulates GAP expression to 50%, but the growth of this clone is normal (data not shown).

We reasoned that the growth inhibition could be due to an aptameric interaction with a cellular protein involving phosphorothioate linkages, since the unmodified antisense oligonucleotide had no effect on cell growth. Because the capped sense oligonucleotide was also without effect, it seems that the effect of the phosphorothioate linkages is sequence specific. Phosphorothioate-capped oligonucleotides in general are not growth inhibitory to K562 cells at the same dose (13, 16). One candidate protein for aptameric interaction was the oncogenic protein tyrosine kinase $p210^{bcr\text{-}abl}$ which is known to protect K562 cells from apoptotic cell death and has been reported to interact with phosphorothioate oligonucleotides containing GGC base triplets (6, 13). However, cellular phosphotyrosine levels were found to be normal, indicating that the activity of $p210^{bcr\text{-}abl}$ was not affected. The identity of the putative target molecule is therefore unclear.

Interestingly, the capped antisense oligonucleotide had little effect on the growth of the cell line HL60, but had significant effects on K562. This is reminiscent of a report of a phosphorothioate oligonucleotide which caused cell death in the cell line BV173 but not LAMA-85, whilst not downregulating the expression of the target gene (18). It is therefore possible that the putative target molecule is more susceptible to aptameric interaction with the oligonucleotide in K562 cells.

The results with HL60 cells clearly indicated that neither the phosphorothioate-capped nor the unmodified antisense oligonucleotide caused downregulation of GAP mRNA levels. This contradicts a previous report in which unmodified oligonucleotides to GAP of the same sequence caused downregulation of GAP mRNA levels in HL60 cells (11). The reason for this difference is unclear.

In conclusion, we have reported a specific oligonucleotide sequence that when presented in the setting of terminal phosphorothioate linkages, specifically inhibits proliferation and causes apoptosis in the CML cell line K562. This is particularly exciting because K562 is known to be very resistant to apoptotic cell death (19). It was shown that the effects were not due to antisense mediated gene downregulation and probably represented aptameric interaction with a cellular protein other than $p210^{bcr\text{-}abl}$. The identity of the target molecule may therefore be of significance for the development of therapeutic agents to combat CML. Furthermore, these results highlight the limitations of phosphorothioates in antisense experiments.

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