The cyclin-dependent kinase inhibitors interact with cyclin-ecdk complexes to arrest mitogen-stimulated transit through the cell cycle, but these proteins have recently been shown to have positive regulatory effects on cyclin-ecdk complex activity as well. Most of the previous work in this area has focussed on the finding that overexpressed p21Waf1/Cip1 causes growth arrest. However, mice lacking p21Waf1/Cip1 showed normal development with no aberrancy in their cell cycles, and antisense p21Waf1/Cip1 has only been shown to prevent cell cycle arrest, leading to the conclusion that the cyclin kinase inhibitors may not be required for cell cycle progression. We found that transfection of several lines of vascular smooth muscle cells with antisense oligodeoxynucleotide specific to p21Waf1/Cip1 correlates with decreased cyclin D1/cdk 4, but not cyclin E/cdk 2, association, yet, unexpectedly, results in dose-dependent inhibition of platelet-derived growth factor-BB-stimulated DNA synthesis and cell proliferation. Our finding that p21Waf1/Cip1 exhibits permissive effects on growth factor-induced vascular smooth muscle cell cycle progression, such that its presence is required for growth factor-induced proliferation, is the first such report and opens up a fertile area of research relevant to diseases involving vascular cell proliferation.

Antisense Transfections—Phosphorothioate antisense oligodeoxynucleotides were synthesized by Oligos Etc. (Wilsonville, OR). The p21Waf1/Cip1 antisense vector was designed around the start codon of rat p21Waf1/Cip1, with the sequence 5'-GACATCCACAGGATCGGACAT-3'. The scrambled random sequence control oligodeoxynucleotide was 5'-TGATCGACGATCGGTATC-3'. The scrambled random sequence control oligodeoxynucleotide was 5'-TGATCGACGATCGGTATC-3'. For the lipofection procedure, cells were grown to 90% confluence, the appropriate concentration of oligodeoxynucleotide was mixed with 6.6 μl of Lipofectin™ per ml of Opti-MEM medium and was added to the cells for 4 h at 37 °C. The cells were washed and serum-free medium (without oligodeoxynucleotide) was added overnight, the medium was changed in the morning and the cells were incubated in serum-free medium for the times indicated.

Western Blots—Cells were grown to confluence in 6-cm culture dishes and serum deprived. After transfection and or treatment with appropriate agonist, the cells were washed with phosphate-buffered saline and lysed in lysis buffer and the supernatant was Western blotted as appropriate agonist, the cells were washed with phosphate-buffered saline and lysed in lysis buffer and the supernatant was Western blotted as described (17).
RESULTS

Recent data has demonstrated that the CKIs, which have long been classified (as the acronym indicates) as growth inhibitors, also function under some conditions as positive regulators of cyclin-cdk complexes. The available studies addressing the role of CKIs in VSM cells have demonstrated negative effects of p21Waf1/Cip1 only on cell cycle progression. We employed antisense techniques to examine the dependence of cell growth on p21Waf1/Cip1 in our VSM cell lines. The oligodeoxynucleotides used were generated around the ATG start codon using GenBank sequences and were screened for lack of stable secondary structures or stable homodimer formation (OligoTech software, Oligos Etc.). We used three independent controls in these experiments: (i) “dummy” transfection with Lipofectin but no DNA; (ii) random sequence oligodeoxynucleotide transfection; and (iii) sense p21Waf1/Cip1 oligodeoxynucleotide transfection. VSM cells were transfected with the appropriate oligodeoxynucleotide or control overnight in serum-free medium, and the next day the cells were stimulated with PDGF-BB and DNA synthesis and cell number were assessed. Significant inhibition of PDGF-stimulated DNA synthesis occurred when the cells were transfected with antisense p21Waf1/Cip1, but not with sense p21Waf1/Cip1, dummy transfection (Fig. 1A), or random sequence control (Fig. 1B). To confirm that the observed growth inhibition was specific to the antisense p21Waf1/Cip1 oligodeoxynucleotide, we performed dose/response analysis. There was inhibition of DNA synthesis with increasing concentration of antisense p21Waf1/Cip1 oligodeoxynucleotide up to 200 nM, with no effect of sense p21Waf1/Cip1 oligodeoxynucleotide (Fig. 1C). To demonstrate that this effect was not specific to the A10 cell line, we demonstrated a similar effect in a bovine VSM cell line (Fig. 1D) and in the A7r5 rat VSM cell line (data not shown). Changes in cell number were shown to parallel the alterations in DNA synthesis (Fig. 2).

To establish whether the oligodeoxynucleotides crossed the cell membrane and entered the nucleus in order to inhibit p21Waf1/Cip1 protein production, cells were transfected with a fluorescein-tagged p21Waf1/Cip1 antisense oligodeoxynucleotide (with the same sequence as the p21Waf1/Cip1 antisense) and were examined for transfection efficiency. Upon examination by fluorescence microscopy, these cells demonstrated 100% transfection efficiency (Fig. 3), as has been reported for this technique (19).

We next asked whether antisense transfection with p21Waf1/Cip1 antisense oligos indeed decreases p21Waf1/Cip1 protein levels. We employed the fact that PMA is a potent inducer of p21Waf1/Cip1 (20, 21) to examine p21Waf1/Cip1 levels after antisense transfection. Since p21Waf1/Cip1 protein levels were induced in VSM cells between 2 and 6 h after PMA stimulation (Fig. 4), we examined p21Waf1/Cip1 levels in transfected cells after similar times of PMA stimulation. After transfection with appropriate oligodeoxynucleotide and subsequent overnight incubation in quiescent media, antisense p21Waf1/Cip1 oligodeoxynucleotide caused significant attenuation of PMA-induced p21Waf1/Cip1 levels in VSM cells up to 6 h (Fig. 5). There was no effect of the p21Waf1/Cip1 sense control oligodeoxynucleotide on cellular p21Waf1/Cip1 levels (compare with Fig. 4).

To check for specificity of protein inhibition by the antisense p21Waf1/Cip1 oligodeoxynucleotide, we examined protein levels of p21Waf1/Cip1 and p27Kip1 after transfection with antisense p21Waf1/Cip1 oligodeoxynucleotide. In these experiments, we assessed the ability of antisense oligodeoxynucleotides to inhibit maximally stimulated CKI expression (see Fig. 4), thus the cells were stimulated with PMA for 4 h at various times after overnight serum starvation. While antisense p21Waf1/Cip1 completely inhibited p21Waf1/Cip1 protein even after maximal stimulation with PMA, there was a slight decrease in p27Kip1 protein as well as with this oligodeoxynucleotide (Fig. 6A). This is likely due to sequence similarity between the two genes, as p21Waf1/Cip1 shares 43% sequence identity with p27Kip1 in the cdk/cyclin-binding site (residues 27–88), located in the conserved N terminus (22, 23). Levels of the VSM cell structural protein α-actin were not altered after transfection under identical conditions (Fig. 6B), demonstrating that the effect of antisense oligonucleotides on cell proteins was not a general inhibitory one. Furthermore, we do not believe that the slight p27Kip1 inhibition is playing a significant role in mitogenic inhibition, in light of data from other investigators (24) as well as our cyclin/cdk data discussed below.

While the CKIs have been shown to be growth inhibitors in VSM cells (2, 3, 25), various CKIs have been reported to act as “assembly factors” in other cells, both in vivo (14) and in vitro (11), yet previous studies have not shown inhibition of growth with interference of cyclin/cdk association. Since the cyclin D1/cdk 4 interaction occurs early after growth factor stimulation (reviewed in Ref. 26) and because this interaction is facilitated by p21Waf1/Cip1 and p27Kip1 in vivo (11), we decided to examine this association as a possible mechanism of the permissive effect on growth of p21Waf1/Cip1 in VSM cells. Because other CKIs, such as p27Kip1, have been shown to affect cyclin/cdk 2 interaction (12, 27), we also examined the nature of this association. Cells were transfected with p21Waf1/Cip1 antisense or sense oligodeoxynucleotide, allowed to grow overnight in serum-free media, and then stimulated for various times with PDGF-BB. The cells were subsequently immunoprecipitated with either cyclin D1 or cyclin E and immunoblotted with cdk 4 or cdk 2, respectively. Antisense p21Waf1/Cip1-transfected cells showed a marked decrease in association of cyclin D1 and cdk 4 at all times of PDGF stimulation, with no change in the cyclin E/cdk 2 interaction (Fig. 7). Thus the inhibitory effect of antisense p21Waf1/Cip1 oligodeoxynucleotide in VSM cells is likely by means of disruption in cyclin D1/cdk 4 interaction and thus prevention of activation of cdk 4 by cyclin D1.

DISCUSSION

Ablerrant proliferation of VSM-like cells is pathogenic for a variety of diseases, such as atherosclerosis and angioplasty restenosis (28), as well as renal mesangial cell proliferation (29), thus the mechanism by which these cells are stimulated to grow will be important in designing antiproliferative therapies for treating these and other diseases. Published studies in VSM cells focus on the antiproliferative action of CKI overexpression (2, 3, 25, 30), and there are even some studies promoting the idea that pharmacological methods to increase p21Waf1/Cip1 may be useful in preventing the VSM cell proliferation seen after coronary angioplasty (31–33). Our data showing for the first time that inhibition of p21Waf1/Cip1 efficiently blocks mitogen-stimulated VSM cell proliferation injects a note of caution in these pursuits, and might also explain the failure to find mutations in the gene encoding p21Waf1/Cip1 in human tumors (34).

While much of the early work on the CKIs has focussed on their role as growth inhibitors, it had been somewhat puzzling that expression of these molecules was increased early after mitogen stimulation (20, 35). This led to more recent data showing the ability of some CKIs to take part in formation of the cyclin/cdk complexes, and thus to serve as assembly factors important for promoting cyclin/cdk association (11, 36). In support of this role for the CKIs, others have shown that assembly of cyclin D1/D2-cdk4 complexes was impaired in fibroblasts from mice lacking the genes encoding p21Waf1/Cip1 and/or p27Kip1 (14), and that both p21Waf1/Cip1 and p27Kip1 actively promoted interaction between the cyclin Ds and their counter-
Fig. 1. Antisense p21\textsuperscript{Waf1/Cip1} oligodeoxynucleotide transfection inhibits VSM cell DNA synthesis in a dose-dependent manner. A10 VSM cells were lipofected with: (a) no DNA, 200 nM sense p21\textsuperscript{Waf1/Cip1} or antisense p21\textsuperscript{Waf1/Cip1} (\(\square\), no DNA; \(\blacksquare\), sense p21; \(\blacklozenge\), anti-p21) and (b) 200 nM random sequence control oligodeoxynucleotide or antisense p21\textsuperscript{Waf1/Cip1} (\(\square\), random sequence; \(\blacklozenge\), anti-p21); and various concentrations of sense p21\textsuperscript{Waf1/Cip1} or antisense p21\textsuperscript{Waf1/Cip1} in A10 (c; \(\square\), sense p21; \(\blacklozenge\), antisense p21) and bovine VSM cells (d; \(\square\), sense p21; \(\blacklozenge\), antisense p21). The cells were placed in serum-free medium overnight and stimulated with PDGF-BB (30 ng/ml) for another 18 h. DNA synthesis was assessed by [\(^3\)H]thymidine incorporation and is expressed as mean ± S.E. of three wells per data point. The absolute counts differ between experiments due to different confluency of the cells. The experiments shown are representative of two to three separate experiments.
part cdk5 by stabilizing this complex (11). However, primary fibroblasts from p21- and p27-null mice did not show overtly aberrant cell cycles, despite the finding by those investigators that overall cyclin D-dependent kinase activity was reduced below the assay limit of detectability, leading these authors to raise the possibility that the CKIs are not required for cell cycle progression (14). Other studies have shown an increased growth rate of p21\(2/2\) as compared with wild type mouse embryonic fibroblasts (37), and no apparent G1 block in human colorectal cancer cells (38). The difference of our data as compared with that in the mouse cells may well be due to cell type, but our finding of growth inhibition in cells lacking active p21\(Waf1/Cip1\), we believe, better explains the “essential activator” role of p21 promulgated by that group (14). Furthermore, since p21\((-/-)\) mice appear to develop normally (37), it is conceivable that p21\(Waf1/Cip1\) disruption only affects “adult” cells, or that redundant pathways for cell growth are not present in A10 cells. Nevertheless, our data is the first showing that the presence of these pleiotropic molecules is required for growth factor-mediated G1 progression in any cell type.

Other investigators have shown that cells transfected with antisense p21\(Waf1/Cip1\) lose their ability to become growth arrested by epidermal growth factor (15). In this case, there was certainly no inhibition of DNA synthesis by the antisense construct, however, these cells are transformed and are thus quite different from our A10 VSM cells. In order to explain this and other extant data in this field, it has been hypothesized that, in the absence of Cip/Kip CKIs, the function of cyclin D/cdk 4 to titrate p21\(Waf1/Cip1\) is no longer necessary, and in the case of experimental absence of these CKIs, cyclin E- and cyclin A-dependent kinases become sufficient to phosphorylate Rb and cause DNA synthesis to proceed (1). This does not appear to be occurring in our VSM cell line, where the absence of p21\(Waf1/Cip1\) leads to significantly diminished responsiveness to PDGF.

While our antisense p21\(Waf1/Cip1\) oligodeoxynucleotide clearly inhibits p21, there is also slight inhibition of p27\(Kip1\) protein as well by this oligodeoxynucleotide (Fig. 3). While this occurrence is likely due to the sequence similarity between the two genes (22, 23), we do not believe that the slight amount of inhibition of p27\(Kip1\) is actively inhibiting VSM cell growth in our experiments, since cyclin E/cdk 2 association is not affected (19, 27, 39) (see Fig. 7). Furthermore, others have reported suppression of quiescence, not of G1-phase progression, in fibroblasts transfected with antisense p27\(Kip1\) (24). In any case, our finding of unchanged α-actin protein levels in antisense p21-transfected cells argues against a general suppressive effect of these oligode-
Very recent work has shown that lack of a functional gene encoding p21Waf1/Cip1 in transgenic mice ameliorates progression of chronic renal failure after partial renal ablation (29). Proliferating cell nuclear antigen was found to be significantly increased in p21\(^{-/-}\) animals, but the degree of mesangial expansion was not quantitated. While the decrease in progression of renal failure was assumed to be due to a more hyperplastic (rather than hypertrophic) reaction in the p21\(^{-/-}\) animals, our new data may shed some light on this phenomenon by suggesting that the response in p21\(^{-/-}\) animals may have been a result of decreased mesangial cell mitogenesis due to inhibition of p21Waf1/Cip1 expression. This point of view is reinforced by others, noting that "all kidney growth parameters reported by Megyesi et al. (29) are lower in p21\(^{-/-}\) mice compared with p21\(^{+/+}\) mice" (40). Indeed, since renal mesangial cells are modified VSM cells, our work could lead to novel approaches to therapy of renal ailments, as well as other proliferative diseases, employing inhibitors of the CKIs.

We believe that a major importance of our work lies in its relevance and potential applications to diseases involving aberrant VSM (or similar type) cell proliferation, such as atherosclerosis, angioplasty restenosis, and renal disease. While the available research on the CKIs in VSM cells has focused on the induction of the Cip/Kip family of CKIs in the presence of antiproliferative situations (25, 31, 41, 42), the inhibitory effects of CKI antisense constructs that we show here may well be specific to vascular-like cells, as they were not observed in A431 cells (15). While much work in the area of inhibition of VSM cell proliferation (including our own) has been concentrated on the elements of the MAP/ERK and other cytoplasmic kinase cascades and their inhibitors, we believe that, in light of our data presented here, vascular biologists may now wish to focus more efforts on events further downstream in the mitogenic signaling cascades (i.e., in the nucleus), such as the cyclin/cdk/CKI signaling systems.

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p21Waf1/Cip1 Is Required for VSM Cell Mitogenesis

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Additions and Corrections

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*p21^{Waf1/Cip1}* is an assembly factor required for platelet-derived growth factor-induced vascular smooth muscle cell proliferation.

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An earlier version of fig. 7 was inadvertently published with this manuscript. The correct figure, with the control lanes as described in the figure legend, appears here.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.