The human MAT1 gene (ménage à trois 1) is an assembly factor and a targeting subunit of cyclin-dependent kinase (CDK)-activating kinase. The novel mechanisms by which MAT1 forms an active CDK-activating kinase and determines substrate specificity of CDK7-cyclin H are involved in the cell cycle, DNA repair, and transcription. Hyperplasia of vascular smooth muscle cells (SMCs) is a fundamental pathologic feature of luminal narrowing in vascular occlusive diseases, and nothing is yet known regarding the cell cycle phase specificity of the MAT1 gene in its involvement in SMC proliferation. To investigate such novel regulatory pathways, MAT1 expression was abrogated by retrovirus-mediated gene transfer of antisense MAT1 RNA in cultured rat aortic SMCs. We show that abrogation of MAT1 expression retards SMC proliferation and inhibits cell activation from a nonproliferative state. Furthermore, we have demonstrated that these effects are due to G1 phase arrest and apoptotic cell death. Our studies indicate a link between cell cycle control and apoptosis and reveal a potential mechanism for coupling the regulation of MAT1 with G1 exit and prevention of apoptosis.

The human MAT1 gene (ménage à trois 1) was discovered as the third subunit of cyclin-dependent kinase (CDK)-activating kinase (CAK) (1–3). CAK exists in either of two forms: (a) the major ternary CAK containing CDK7, cyclin H, and MAT1, which is assembled by MAT1 in the absence of activating phosphorylation of the T-loop of CDK7; and (b) the minor binary CAK lacking MAT1, which requires the phosphorylation of the T-loop of CDK7 for CDK7 binding to cyclin H in the absence of MAT1 (1, 4, 5). MAT1 is associated with both a free form of CAK and a transcription factor IIH (TFIIH)-bound form of CAK (1, 4, 6–10). Up to the present, the known functions of MAT1 have been associated with CAK. CAK was originally implicated in cell cycle control by its ability to phosphorylate and activate CDK1, CDK2, and CDK4 (11–16). Subsequently, CAK was found to associate with TFIIH and to phosphorylate the carboxyl-terminal domain of RNA polymerase II (1, 4, 7–10). TFIIH is required for both initiation of RNA polymerase II-catalyzed transcription and nucleotide excision repair (17–19). Thus, the concept that CAK functions in the regulation of the cell cycle, DNA repair, and transcription has emerged.

Interestingly, all of the functions that link CAK to the cell cycle, DNA repair, and transcription are mediated by MAT1, i.e., MAT1 activation of CAK by stabilizing the association of CDK7 with cyclin H (1, 4, 6) and MAT1 determination of substrate specificity of CAK through (a) switching the substrate preference of CAK to the carboxyl-terminal domain over CDK in the presence of MAT1 (20) and (b) the requirement of MAT1 for efficient phosphorylation of the tumor suppressor protein p53 by CDK7-cyclin H (21). The efficiency of bipartite CDK7-cyclin H in CDK2 phosphorylation is not affected by the addition of MAT1 (20, 21), suggesting that MAT1 more likely acts as a targeting subunit of CAK than significantly influencing CDK7-cyclin H phosphorylation of CDKs. TFIIH lacking the CAK subcomplex completely recovers its transcriptional activity in the presence of free ternary CAK; MAT1 interacts with essential components of the DNA repair machineries XPD (ERCC3) and XPD (ERCC2), which are two helicase subunits of TFIIH that mediate the association of CAK with core TFIIH (10, 22, 23). However, these studies have provided little information about the cell cycle phase specificity and the corresponding regulatory mechanisms of MAT1 itself in the control of cell proliferation.

It is known that the fundamental feature of vascular occlusive disease, including atherosclerosis, hypertension, restenosis, and transplant arteriopathy, is an accumulation of cells and extracellular matrixes in the intima, which consequently results in the narrowing of the vascular lumen. The vascular smooth muscle cell (SMC) represents the cell type most often implicated in the process of luminal narrowing (24–31). In recent years, rapid progress has been made in identifying and understanding the functions of some cell cycle regulators in controlling SMC proliferation. Many important cell cycle factors, including the catalytic subunit CDC2 (32), the negative cell cycle regulator retinoblastoma protein (pRb) (29, 33), and the CDK inhibitor p21 (34), have shown to play important roles in regulating SMC proliferation, even though the molecular mechanisms still remain to be further studied. Considering that eukaryotic cell proliferation is a process that is highly regulated by the ordered assembly and determined substrate specificity of CDKs (35, 36), the functions of MAT1 in assembling and determining substrate specificity of CAK (20, 21) may
be directly involved in the regulation of a specific cell cycle phase. Given that MAT1 functions in the cell cycle as an assembly factor and a targeting subunit of CAK, we intended to determine the cell cycle phase specificity of MAT1 functions and the corresponding mechanism of the regulation of SMC proliferation. Our studies show that MAT1 is required for G1 exit. A loss of MAT1 arrests SMCs in G1 phase and induces apoptosis to kill these arrested cells.

EXPERIMENTAL PROCEDURES

Retroviral Vectors—An N-terminal 462-bp fragment of the MAT1 gene starting from the 5'-end, 40 bp upstream of the ATG initiation codon and extending to +422 bp of the coding region, was cloned into retroviral vector G1xSvNa (Genetic Therapy Inc./Novartis) in the antisense orientation (G1AsMatSvNa). G1AsMatSvNa indicates the order of the promoter and coding region of the constructs (G1, Moloney murine leukemia virus long terminal repeat sequences; AsMat, antisense MAT1 RNA fragment; S, SV40 early enhancer and promoter; Na, neomycin phosphotransferase). The G1AsMatSvNa construct was confirmed by sequencing as described previously (37, 38) using primers flanking the neomycin phosphotransferase. The G1AsMatSvNa construct was transduced from the described construct as described (37) and the expression level of MAT1 was used as a positive control. The expression level of MAT1 was determined by scanning MAT1 content using a ScanJet IICXT densitometer (Hewlett-Packard Co.).

Cell Proliferation Analysis—Cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cultures were used for determining the ratio of cell duplication. The same number of cells were plated in 24-well plates. 24 h after plating, the cells were counted for 3 consecutive days before reaching confluence.

To determine the number of living cells in culture, a cell proliferation assay kit (Promega) was used as recommended by the manufacturer. Briefly, the same number of cells were plated in 96-well plates and cultured for 72 h to 90% confluence. Cells were incubated with MTS solution (Promega) and the formazan product as measured by the absorbance at 490 nm using a Microplate Reader (Molecular Devices) is directly proportional to the number of living cells in culture.

Flow Cytometric Analysis of Cell Cycle Status and Apoptosis—Cell cycle status and cell cycle phase specificity of apoptosis were determined by simultaneously measuring DNA content and DNA breaks as described (41) with minor modifications. Cells were grown in 12-well plates to confluence (when they exhibited contact inhibition) and then scrapped with a 200-μl pipette tip to create a 1-mm track devoid of cells in the central area of the wells. The “wound” tracks were immediately washed to remove the detached cells, and fresh medium was added. 24 h after the wound tracks were created, G1xSvNa retroviral constructs bearing a nuclear targeting β-galactosidase were added at a multiplicity of infection of 10 in the presence of Polybrene (8 μg/ml) for 2 h. 48 h post-transduction, gene transfer efficiency was measured by determining the percentage of β-galactosidase-positive cells upon exposure to 1 μl of staining solution (5 mg potassium ferricyanide, 5 mg potassium ferrocyanide, 2 mg MgCl₂, and 400 μg/ml X-gal (GIBCO BRL)). The number of blue cells, which reflected the activation of SMC proliferation and the number of cell divisions, was counted for 3 consecutive days before reaching confluence.
western analysis. MAT1 content in the transduced stable clones was determined by Western analysis. 10 μg of cell lysate proteins from stable clones as well as from nontransduced (blank) cells were separated on 16% SDS-polyacrylamide gel. The expression level of 37-kDa MAT1 protein was detected by polyclonal anti-MAT1 antibodies. The 40-kDa recombinant His-MAT1 protein (Rec.MAT1) served as a positive control. A, MAT1 expression level before the experiments; B, MAT1 content 5 months later after a series of experiments.

with 0.015% propidium iodide (Sigma) at room temperature for 30 min; and examined under epifluorescence illumination at ×400 magnification. The morphologic apoptotic changes of cells without propidium iodide staining were monitored under a phase-contrast microscope at ×100 magnification.

In Situ Detection of Apoptosis Using Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL)—In situ DNA fragmentation was detected using a TUNEL reaction kit (Boehringer Mannheim) following the manufacturer’s instructions with some modifications. In brief, cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (vector blank) cultures were grown to ≈80% confluence on 8-well chamber slides, fixed in 4% paraformaldehyde solution for 30 min at room temperature, permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice, washed twice with PBS, and incubated with 50 μl of TUNEL reaction mixture in a humidified chamber for 1 h at 37 °C. Negative controls for blank, vector, and MAT1-AS cells were incubated with reaction buffer lacking terminal deoxynucleotidyltransferase. The slides were washed three times with PBS and stained with 0.015% propidium iodide for 30 min at room temperature. After a final wash with PBS, the slides were air-dried and mounted under glass coverslips with aqueous medium.

The apoptotic cells in each field were examined with a Zeiss epifluorescence microscope (Axioplan/MC100) at 100 magnification. The morphologic apoptotic changes of cells without propidium iodide staining were monitored under a phase-contrast microscope at ×100 magnification.

Analysis of DNA Fragmentation—Low molecular weight DNA of the soluble cytoplasmic fraction was isolated as described (43–45). ~1.5 × 10^6 cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cultures were lysed in 0.5 ml of hypotonic buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.5% (v/v) Triton X-100 on ice for 30 min and centrifuged at 27,000 × g for 15 min at 4 °C. RNA in the supernatant was degraded by incubation with DNase-free RNase (50 μg/ml) at 37 °C for 1 h, followed by proteinase K digestion. DNA was precipitated with 0.1 volume of 3 mM sodium acetate, pH 5.2, 20 μg/ml glycogen, and 2 volumes of anhydrous ethanol at −20 °C for 20 min. The same amount of DNA (10 μg) was electrophoresed through agarose gel, stained with ethidium bromide, and visualized under UV light. A 100-bp DNA ladder was used as a molecular size marker.

RESULTS

MAT1 Expression Is Inhibited by RNA Antisense Sequence—To examine whether retrovirus-mediated gene transfer of antisense MAT1 RNA inhibits endogenous MAT1 expression, the cellular MAT1 content from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cultures was determined by Western blotting. The results show that the MAT1 content was reduced ~70% in MAT1-AS-transduced cells (Fig. 1A). To ensure that the phenotype of MAT1 expression was consistently maintained during the experimental period, MAT1 content was examined again 5 months later, after finishing all of the testing (Fig. 1B). The data confirmed that >50% decreased MAT1 expression in MAT1-AS cells was maintained during the experimental period.

Abrogation of MAT1 Inhibits Aortic SMC Proliferation and Activation—To examine whether the abrogation of MAT1 affects SMC proliferation, we used three different approaches. First, we sought to test whether abrogation of MAT1 inhibits cell duplication. This was accomplished by monitoring cell numbers over a 3-day culture period. The same number of cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cultures were plated at t = 0 h and counted at 24, 48, and 72 h. The cell number from all groups declined ~20% at 24 h because of natural attachment of the cells. The data show that cell duplication was at least 50% inhibited in the MAT1-AS culture.
compared with the blank and vector controls (Fig. 2A).

Our second approach was to determine the proliferating cell ratio in culture. The same number of cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cultures were plated and grown for 72 h for proliferation assay. The results show that the number of living cells, represented by the amount of bioreduced formazan, was reduced ~50% in the MAT1-AS culture compared with the blank and vector controls (Fig. 2B).

Arterial SMCs in vivo are normally maintained in a nonproliferative state within the tunica media. Upon arterial injury, activated SMCs migrate into the intimal layer of the arterial wall, where they proliferate and produce extracellular matrix components, which leads to neointima formation.
Abrogation of MAT1 arrests cells in G1 phase and induces apoptosis. Nuclei of cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cells were analyzed for DNA content and DNA breaks by flow cytometry. The estimated proportion of G0/G1 phase cells in the negative control for blank (lacking terminal deoxynucleotidyltransferase), blank, and vector cultures (A–C, respectively) ranged from 32 to 35%. In contrast, 66% of the MAT1-AS cells were in G0/G1 phase (D). DNA breaks were not detectable in the blank.
and causes angioplasty failure (25, 28, 46). Our third approach was to test whether the abrogation of MAT1 inhibits the activation of SMC proliferation from a nonproliferative state. Cells from G1AsMatSvNa (MAT1-AS)-transduced, G1xSvNa (vector)-transduced, and nontransduced (blank) cultures were grown to confluence and then scraped to release cells from contact inhibition. 24 h after the wound tracks were created, retrovirus-mediated β-galactosidase gene transfer was performed to test the activation of SMC proliferation. Cell proliferation along the wound margin was markedly inhibited (Fig. 3A), as evidenced by the fact that only one positive X-gal-stained cell was detected in the MAT1-AS culture. Also, the closure of the wound track was significantly inhibited in the MAT1-AS cultures (Fig. 3B, MAT1-AS panel IV) compared with the blank (Blank panel IV) and vector (Vector panel IV) cultures, in which the wound tracks were completely closed at t = 60 h. In contrast, the time for closing the wound track in the MAT1-AS culture was t = 120 h (data not shown). These results show that proliferation and migration were inhibited in MAT1-AS cells by ~50%. Our data strongly suggest that MAT1 expression is required for the activation of SMC proliferation and migration from a nonproliferative state.

Abrogation of MAT1 Arrests Cells in G₁ Phase and Triggers Apoptosis—Since our preliminary data (data not shown) and some previous studies (20, 21) indicate that CDK7-cyclin H phosphorylation of CDKs may be MAT1-independent, we linked the cell cycle function of MAT1 using cell cycle analysis rather than testing whether the reduced MAT1 expression affects CAK-mediated phosphorylation of CDKs in these studies. Our initial studies showed that RNA antisense abrogation of MAT1 inhibited cell proliferation and activation; we sought to determine whether this inhibition was due to disruption of the normal cell cycle regulation and/or induction of apoptosis. To answer these questions, we quantified the effect of MAT1 abrogation on the cell cycle and apoptosis in relation to their position in the cell cycle. The cell cycle status and apoptosis of G1AsMatSvNa (MAT1-AS)-transduced SMCs versus controls of G1xSvNa (vector)-transduced and nontransduced (blank) SMCs were measured by flow cytometry (Fig. 4). As shown in Fig. 4 (A–C), the negative controls for blank (lacking terminal deoxynucleotidyltransferase), blank, and vector cells showed normal cell cycle profiles with 32–35% of the cells in G₀/G₁ phase, 39–46% in S phase, and 22–27% in G₂/M phase, and the total of replicating and dividing cells was 61–73%. However, the cells from the MAT1-AS culture showed 66% of the cells in G₀/G₁ phase, 21% in S phase, and 13% in G₂/M phase (Fig. 4D), and the total of replicating and dividing cells was 54%. Also, a peak of less than 2N DNA content falling in front of G₀/G₁ phase represented DNA degradation, an event reminiscent of apoptosis in the MAT1-AS culture (Fig. 4D). By comparing MAT1-AS cells with controls, the data show that in MAT1-AS cells, 1) twice as many cells were arrested in G₁ phase, 2) replicating and dividing cells decreased ~50%, and 3) DNA degradation occurred during the cell cycle. We further analyzed the apoptotic cell ratio and its position in the cell cycle using the dual parameter display method (Fig. 4, E–H). The results show apoptotic cell death to be undetectable in the negative controls for blank (lacking terminal deoxynucleotidyltransferase), blank, or vector cultures (Fig. 4, E–G). In contrast, apoptotic cell death occurred ~42% of the cells in MAT1-AS cultures (Fig. 4H), and the majority of these cell deaths occurred in arrested G₁ cells. These results strongly suggest that MAT1 is required for G₁ exit and that apoptosis is triggered by G₁ arrest.

Apoptotic Morphologic Criteria in Cells Transduced with Antisense MAT1 RNA—G1AsMatSvNa (MAT1-AS)-transduced cells not only showed inhibited cell proliferation, but also had a visible apoptotic morphology (Fig. 5). The cells became rounded and vector cultures (F and G, respectively). An estimated 42% DNA breaks occurred in the MAT1-AS cells, and the majority of DNA breaks consisted of arrested G₁ phase cells (H).
and developed vesiculations around the cell borders (Fig. 5C) compared with nontransduced (blank) and G1xSvNa (vector)-transduced cells (Fig. 5, A and B). We also used the propidium iodide stain to analyze the apoptotic morphologic changes in the nuclei of the cells. Under an epifluorescence microscope, we found condensation of nuclear chromatin and nuclear fragmentation in MAT1-AS cells (Fig. 5F). Similar apoptotic changes were undetectable in blank (A, D, and G) and vector (B, E, and H) cells. Negative controls for blank, vector, and MAT1-AS cells were incubated with reaction buffer lacking terminal deoxynucleotidyltransferase (data not shown).

**DISCUSSION**

Cell cycle transitions are processes that are highly regulated by ordered assembly and timing-activated CDK complexes, and inhibition of these kinases will cause cell cycle arrest (35, 36). CAK regulates cell cycle progression by activating CDK complexes through phosphorylation of a critical threonine residue in their T-loop domain (50, 51), whereas MAT1 functions in the cell cycle by stabilizing the association of CDK7 with cyclin H (1, 4, 6) and determining substrate specificity of CDK7-cyclin H (20, 21, 23). In contrast to the detailed mechanisms of regulatory subunit cyclins and catalytic subunit CDKs, whose functions have been relatively defined in a certain period of the cell cycle, little is known about the cell cycle phase specificity of the

**Detection of Apoptotic Cell Death**—The biochemical hallmark of apoptosis is the cleavage of chromosomal DNA into oligonucleosomal units (47–49). To characterize this cell death process, the TUNEL assay was used to detect in situ apoptotic cell death. As shown in Fig. 6, the nuclear fragmentation was easily identified by co-locating apoptotic nuclei of G1AsMatSvNa (MAT1-AS)-transduced cells under an epifluorescence microscope using three different filters: red for total DNA stain (A–C), yellow green for apoptotic nuclei stain and red for integral DNA stain (D–F), and green for apoptotic nuclei stain (G–I). The apoptotic nuclei were identified in the MAT1-AS culture (C, F, and I) (arrowhead; bar = 30 μm), but were undetectable in the blank (A, D, and G) and vector (B, E, and H) cells. Negative controls for blank, vector, and MAT1-AS cells were incubated with reaction buffer lacking terminal deoxynucleotidyltransferase (data not shown).
targeting subunit MAT1 in cell cycle regulation. In this study, we have shown that abrogation of MAT1 arrests SMCs in G₁ phase, triggers apoptosis, and retards SMC proliferation. Thus, our data suggest a novel mechanism by which MAT1 controls cell proliferation through regulating the G₁ exit and mediating apoptosis.

**MAT1 Regulates G₁ Exit to Control Cell Proliferation—**G₁, S, G₂, and M phases are major cell cycle states. Identifying the factors that trigger the transitions between cell cycle phases is one of the major goals in cell cycle research (35, 36, 50–53). In our studies using RNA antisense abrogation of MAT1, we found that the proportion of G₁ phase cells is doubled and that cells in S and M phases decrease 50% (Fig. 4D) compared with controls (Fig. 4, A–C). These data, showing that abrogation of MAT1 causes G₁ arrest, reduces proliferating cells, and induces DNA degradation (Fig. 4, A–D), are consistent with the results of cell proliferation assays. For example, SMC proliferation is decreased ~50% in antisense MAT1-transduced cells (Fig. 2); SMC proliferation efficiency from a nonproliferative to proliferative state is inhibited at least 50% (Fig. 3). It is known that cell cycle transition through the G₁ restriction point and entry into S phase are controlled by the activities of CDK complexes (35, 36, 51). Our results strongly suggest that abrogation of MAT1 may cause failure of assembly and/or determination of substrate specificity of CK/ or other CDK complexes at the G₁ exit, so that the cells are arrested in G₁ phase. Currently, a CDK4-cyclin D complex has been depicted downstream of CAK (11, 12, 16). The CDK4-cyclin D complex phosphorylates and inactivates pRb for G₁ exit (54, 55). A loss of cyclin D-dependent kinase activity prevents many cultured cell lines from entering S phase (56), whereas overexpression of cyclin D shortens the G₁ phase (57). Besides the CDK4-cyclin D complex, CDK2-cyclin E and CDK2-cyclin A complexes are also involved in G₁/S transition (58, 59). Given that a loss of MAT1 causes G₁ phase arrest, our data support the view that MAT1 functions in G₁ exit to control G₁/S transition. The challenging question that awaits to be answered is how the G₁ exit is regulated by MAT1: (a) by determining substrate specificity of CK/ at G₁/S transition through directly interacting with those known CDK complexes of G₁/S transition, e.g., cyclin D-, A-, or E-associated CDK complexes; (b) by regulating other CDK complex assembly and interaction; or (c) by modification of substrate specificity before the restriction point or at G₁/S transition, e.g., whether pRb or p53 is one such target.

**Abrogation of MAT1 Triggers Apoptosis—**Apoptosis removes damaged, virus-infected, and unwanted cells for (i) development and homeostasis, (ii) defense, and (iii) aging (60–62). Under normal circumstances, if any damage or block is irreversible, most cells initiate a sequence of biochemical events leading to programmed cell death or apoptosis (63). In our studies, apoptotic cell death was induced by G₁ phase arrest (Fig. 4D), and the majority of apoptosis occurred in the arrested G₁ cells (Fig. 4H). Our data also show that the retarded cell proliferation (Figs. 2 and 3) is the consequent incident following G₁ phase arrest and apoptotic cell death. Control of cell number is determined by an intricate balance of cell death and cell proliferation. Our data not only demonstrate that the inhibited SMC proliferation was due to G₁ arrest and apoptosis, but also indicate a link between cell cycle control mechanisms and apoptosis. A recent report that an antitumor agent, Noscaphine, arrests cells at mitosis and induces apoptosis is an example that apoptosis machinery responds to disregulated cell cycle events and kills dividing cells (45). Some cell cycle components, e.g., p34CDC2, cyclin B-, and cyclin A-associated CDKs, may participate, directly or indirectly, in part of the apoptotic pathway under certain conditions (64–67). Our results, together with other studies, support the view that the MAT1 gene, as a G₁ exit factor to trigger G₁/S transition, may also act as an upstream regulator of apoptosis machinery for homeostatic balance of cell population. Determining how the abrogation of MAT1 causes G₁ phase arrest and induces apoptosis may elucidate the mechanisms that link cell cycle control with apoptosis and allow the design of therapies that prevent cells from replication and that enhance cell death in the treatment of luminal narrowing in vascular occlusive diseases and proliferation disorders in cancer.

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Abrogation of MAT1 Induces G₁ Arrest and Triggers Apoptosis

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