Role for Mammalian Neutral Sphingomyelinase 2 in Confluence-induced Growth Arrest of MCF7 Cells*

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Recently, we reported that neutral sphingomyelinase 2 (nSMase2) functions as a bona fide neutral sphingomyelinase and that overexpression of nSMase2 in MCF7 breast cancer cells caused a decrease in cell growth (Marchesini, N., Luberto, C., and Hannun, Y. A. (2003) J. Biol. Chem. 278, 13775–13783). In this study, the role of endogenous nSMase2 in regulating growth arrest was investigated. The results show that endogenous nSMase2 mRNA was up-regulated ~5-fold when MCF7 cells became growth-arrested at confluence, and total neutral SMase activity was increased by 119 ± 41% with respect to control. Cell cycle analysis showed that up-regulation of endogenous nSMase2 correlated with G0/G1 cell cycle arrest and an increase in total ceramide levels (2.4-fold). Analysis of ceramide species showed that ceramide caused selective increases in very long chain ceramide C24:1 (370 ± 54%) and C24:0 (266 ± 81%) during arrest. The role of endogenous nSMase2 in growth regulation and ceramide metabolism was investigated using short interfering RNA (siRNA)-mediated loss-of-function analysis. Down-regulation of nSMase2 with specific siRNA increased the cell population of cells in S phase of the cell cycle by 59 ± 14% and selectively reverted the effects of growth arrest on the increase in levels of very long chain ceramides. Mechanistically, confluence arrest also induced hypophosphorylation of the retinoblastoma protein (6-fold) and induction of p21WAF1 (3-fold). Down-regulation of nSMase2 with siRNA largely prevented the dephosphorylation of the retinoblastoma protein and the induction of p21WAF1, providing a link between the action of nSMase2 and key regulators of cell cycle progression. Moreover, studies on nSMase2 localization in MCF7 cells showed that nSMase2 distributed throughout the cells in subconfluent, proliferating cultures. In contrast, nSMase2 became nearly exclusively located at the plasma membrane in confluent, contact-inhibited cells. Hence, we demonstrate for the first time that nSMase2 functions as a growth suppressor in MCF7 cells, linking confluence to the G0/G1 cell cycle check point.

Neutral sphingomyelinases (N-SMases) are major intracellular regulators of ceramide, an increasingly recognized bioac-

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The abbreviations used are: N-SMase, neutral sphingomyelinase; nSMase1 and -2, neutral sphingomyelinase 1 and 2, respectively; CDK, cyclin-dependent kinase; GFP, green fluorescent protein; HDF, human diploid fibroblasts; hnSMase2, human nSMase2; mnSMase2, mouse nSMase2; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose)-polymerase; pRb, retinoblastoma protein; siRNA, short interference RNA; SMase, sphingomyelinase; TNF-α, tumor necrosis factor; TRITC, tetramethylrhodamine isothiocyanate; FBS, fetal bovine serum; RT, reverse transcriptase; MS, mass spectrometry.

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C2-ceramide induced significant dephosphorylation of pRb, with increased association of pRb and the E2F transcription factor into a transcriptionally inactive complex (23). In addition, Lee et al. (22) showed that endogenous ceramide synthesized de novo during cell cycle progression might function as an endogenous modulator of pRb in WI-38 HDF cells.

Moreover, evidence was provided for a role for ceramide-activated protein phosphatase, which comprises both protein phosphatase 1 and protein phosphatase 2A, in the dephosphorylation of pRb. Purified protein phosphatase 1 is known to act on pRb, and ceramide was shown to activate protein phosphatase 1 in vitro, leading to dephosphorylation of pRb (24, 25).

Also, in WI-38 HDF, the effects of exogenous ceramide on pRb dephosphorylation were prevented by cotreatment of cells with inhibitors of ceramide-activated protein phosphatase (protein phosphatase 1 and 2A) (26). Thus, and at exogenous ceramide, a pathway has been defined by which ceramide activates protein phosphatases, leading to dephosphorylation of pRb and to subsequent cell cycle arrest.

Differential regulation of cyclin-dependent kinases (CDKs) by ceramide provides an additional mechanism of regulating pRb protein phosphorylation and cell cycle progression. Early reports showed that exogenous ceramide resulted in a dose-dependent induction of the p21WAF1 gene, a major inhibitor protein for CDKs (23, 27–30). Lee et al. showed that in WI-38 HDF cells, exogenous ceramide induced an increase in the levels of p21WAF1, leading to a greater association of p21WAF1 with CDK2, a specific regulator of the G1/S cell cycle check point. In addition, exogenous ceramide was shown to induce selective dephosphorylation of CDK2 and inhibition of its activity (26). Supporting these observations, stimulation of Jurkat cells with docosahexaenoic acid induced an increase in ceramide levels and p21WAF1 levels (31).

Additional emerging evidence points to possible roles of N-SMases in cell cycle regulation. For example, in Molt-4 leukemia cells, serum withdrawal caused activation of a N-Smase, significant elevation in ceramide levels, and arrest in cell cycle progression at G1/S (10). Increases in N-SMase activity and ceramide levels were also observed in WI-38 HDF as they entered the senescent phase (13). An activation of N-Smase by daunorubicin may also be involved in daunorubicin-induced cell cycle arrest and telomerase inhibition (16).

One major limitation in more specific elucidation of the roles of N-SMases in cell cycle regulation has been the absence of molecular tools to study these enzymes. However, recently, two genes, nSMase1 and nSMase2, were identified as candidate N-SMases. nSMase1 was identified by distant homology to bacterial SMases, and nSMase2 was recently identified as a homologue of nSMase1 (33). Whereas careful examination revealed that nSMase1 most likely functions endogenously as a lyso-platelet-activating factor phospholipase C (34), evidence was provided that nSMase2 indeed can function as a sphingomyelinase in cells. Overexpression of nSMase2 caused an increase in the levels of ceramide and a decrease in the levels of sphingomyelin (35).

In that study, it was also noted that overexpressing nSMase2 in MCF7 cells resulted in a significant decrease in cell growth (30%) as the cells reached the plateau/confluence phase (35). Interestingly, an independent line of investigation had also suggested a possible role of nSMase2 in cell contact inhibition. In that study, nSMase2 was identified as a confluent 3Y1 cell-associated gene (cca1) in 3Y1 cells, whose mRNA levels were increased in growth-arrested confluent 3Y1 rat cells (36).

In light of these results, we here investigate the hypothesis that nSMase2 functions as an endogenous regulator of cell growth. Therefore, the goals of this study were to determine the regulation and physiological role of nSMase2 during cell growth and to determine candidate downstream targets for nSMase2. The results demonstrate that endogenous nSMase2 levels are induced upon confluence and that down-regulation of nSMase2 prevents confluence-induced cell cycle arrest. Interestingly, nSMase2 seems to differentially regulate the levels of long chain (C16:0) and very long chain (C24:1 and C24:0) ceramides. The possible mechanisms involved in nSMase2-induced cell cycle arrest during cell confluence are discussed.

EXPERIMENTAL PROCEDURES

Materials—[1-Choline-methyl-3H]Sphingomyelin was provided by Dr. Alcia Bielawska (Medical University of South Carolina, Charleston, SC). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Scintillation mixture Safety Solve was from Research Products International. Other chemicals were from Sigma. Anti-mouse and anti-rabbit TRITC secondary antibody was from Molecular Probes, Inc. (Eugene, OR). Oligodeoxynucleotides were purchased from IDT, Inc. Culture mediums were obtained from Invitrogen.

Cell Lines and Culture Conditions—MCF7 cells transfected with vector alone (MCF7/vector) and those transfected with nSMase2 (MCF7/nSMase2) were maintained in RPMI 1640 and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO2 incubator. The neuroblastoma SK-NSH cell line was maintained in minimal essential medium containing 10% FBS. Young WI-38 HDF (NIA Aging Cell Repository catalog no. AG06814E) were grown in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose and 10% FBS. In experiments investigating the effects of growth arrest, cells were grown under conditions that achieved growth arrest by allowing cells to reach confluence. Thus, cells were grown at a density of 0.4–0.6 × 106 cells in a 100-mm plate to be used the day after for the experiments. Subconfluent growing cells were designated day 2 cells. Confluent arrested cells were collected on the day of confluence or 2 days postconfluence and were designated day 4 and day 6 cells, respectively. Fresh medium was supplemented every 2 days, and cells were removed from the plates by trypsinization. Phase of growth was estimated by counting cells using a hemocytometer as described (35) and by flow cytometric analysis.

RNA Interference Transfection—Synthetic sense and antisense oligonucleotides were purchased from Qiagen (Lafayette, CO). For design of short interfering RNA (siRNA) oligonucleotides targeting nSMase2, a DNA sequence of the type AA (N19) was selected for the human (AAggtaggtctggttgac) and mouse nSMase2 (AAgtagttacgtggttgac). This sequence corresponded to nucleotides 78–96 located 3′ to the first nucleotide of the start codon of the human and mouse nSMase2 cDNA. The DNA sequence was submitted to a BLAST search against the human genome sequence to ensure that only the nSMase2 gene was targeted. A nonspecific siRNA was used as control. Gene silencing experiments. Transfections were done using Oligo-fectAMINE (Invitrogen) as recommended by the manufacturer. Cells were supplemented with a fresh medium each 2 days, and the final siRNA concentration was 166 nM. At the indicated time points after transfection, cells were used for flow cytometric analysis, N-SMase activity assays, G1/S cell cycle measurements, and immunoblotting analysis.

Preparation of Cell Lysates—Cells were lysed by syringe passage in buffer containing 25 mM Tris (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each chymostatin, leupeptin, antipain, and pepstatin A, and post-nuclear lysate (700–800 µl) was used for incubation in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), and 2 mM magnesium acetate for 1 min at 90 °C, followed by 1 h at 37 °C. Cells were plated in 100-mm dishes at a density of 0.4 × 106 MCF7 cells/dish to be used the subsequent day for gene silencing experiments. Transfections were done using Oligo-fectAMINE (Invitrogen) as recommended by the manufacturer. Cells were supplemented with a fresh medium each 2 days, and the final siRNA concentration was 166 nM. At the indicated time points after transfection, cells were used for flow cytometric analysis, N-SMase activity assays, G1/S cell cycle measurements, and immunoblotting analysis.

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N-SMase Activity—Proteins (100 µg) from wild type MCF7 cells or nSMase2 overexpressors (10 µg) were added to 100 µl of reaction mixture containing 100 mM Tris (pH 7.4), 10 mM MgCl2, 0.2% Triton X-100, 10 mM dithiothreitol, and 100 µM [1-Choline-methyl-3H]Sphingomyelin (10 cpm/µmol) and phosphatidylserine (6.7 mol %). The final volume was adjusted to 200 µl with 50 mM Tris buffer (pH 7.4). After 30 min incubation at 37 °C, the reaction was terminated by the addition of 1.5 ml of chloroform/methanol (2:1), the phases were separated by the addition of 200 µl of water, and 400 µl of the upper phase was mixed with 4 ml of Safety Solve (Research Products International) for liquid scintillation counting.
Real Time RT-PCR—mRNA from MCF7 cells was isolated using the Qiagen minikit for mRNA extraction. Complementary DNA was synthesized from 5 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo(dT)12-18. Real time RT-PCR was performed on a PE Biosystems Gene Amp 5700 Sequence Detection System (Foster City, CA). All reaction components were purchased from PE Biosystems. Standard reaction volume was 10 μl and contained 1X SYBR RT-PCR buffer; 3 mM MgCl2; 0.2 μM each dATP, dCTP, and dGTP; and 0.4 μM each primer. Initial steps of RT-PCR were 2 min at 50 °C for UNG erase activation, followed by a 10-min hold at 95 °C. Cycles (n = 40) consisted of a 15-s melt at 95 °C, followed by a 1-min extension at 60 °C. The final step was performed for 1 min. All reactions were performed in triplicate. Threshold for cycle of threshold (Ct) analysis of all samples was set at 0.15 relative fluorescence units. The data were normalized to an internal control gene, β-actin, to correct for RNA preparation.

Data Analysis—Real time RT-PCR results were analyzed using Q-Gene® software (38), which expresses data as mean normalized expression. Mean normalized expression is directly proportional to the amount of RNA of the target gene (nSMase2) relative to the amount of RNA of the reference gene (β-actin).

Primer Design—Primers for β-actin and nSMase2 were designed using the PerkinElmer Primer Express® software. These primers were made to be intramolecular to preclude amplification of genomic DNA. Primer sequences were as follows: β-actin (forward, 5′-ATTGGGCAAT-GAGGCGTTCCC-3′; reverse, 5′-GTTAGTTTCTGAGGACACCA-3′), nSMase2 (forward, 5′-CAACAATGTGAAGCAGATGCCC-3′; reverse, 5′-CGATTCTTGTGGCTAGGTTG-3′).

Cell Cycle Synchronization—Exponentially growing MCF7/control and MCF7/nSMase2 cells were arrested in G2/M phase by incubation in 0.1% (v/v) FBS-supplemented RPMI 1640 for 72 h. To release the cell template; and 500.002 units of AmpErase UNG erase enzyme; 0.35 liters of cDNA

RESULTS

Effects of Overexpression of nSMase2 on the Cell Cycle—In a previous study, we showed that overexpression of nSMase2 in MCF7 breast cancer cells increased N-SMase activity by 20–30 times and caused a decrease in cell growth when compared with the vector-transfected cells (35). Thus, it became important to determine whether this overexpression affected the viability and/or cell cycle progression.

To determine whether nSMase2 might modulate cell cycle progression, the cell cycle was analyzed by flow cytometry in synchronized MCF7/nSMase2 and MCF7/vector control cells. Fig. 1, A and B, shows similar cell cycle distribution of MCF7/vector and MCF7/nSMase2 cells when the cells were in the exponential phase of growth. After 72 h of incubation in 0.1% serum medium, 70% of MCF7/nSMase2 and MCF7/vector cells were arrested in the G2/M phase of the cell cycle. As shown in Fig. 1C, 24 h after stimulation with 10% serum, the distribution of the MCF7/vector cells resembled that of normal exponentially growing cells, with 46 ± 3% of the cells in G2/M phase. However, the MCF7/nSMase2 cells were at least partly retained in the G2/M phase of the cell cycle, with 63 ± 5% of cells in G2/M and 38 ± 2% in S phase. Figure 1D shows that there was a significant difference in the G2/M phase of the cell cycle between overexpressors and control cells. Analysis of PARP degradation by Western blotting (Fig. 1E) showed that overexpression of nSMase2 did not cause any appreciable cell death. Moreover, evaluation of trypan blue-positive cells showed that overexpression of nSMase2 did not cause any appreciable cell death (data not shown). These results demonstrate that overexpression of nSMase2 induces cell cycle arrest

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at G₀/G₁, thus excluding death by either apoptotic or nonapoptotic pathways.

Changes in nSMase2 Expression during Cell Growth Arrest—To determine whether endogenous nSMase2 is regulated during cell growth, nSMase2 expression was studied at various phases of cellular growth. Fig. 2A shows that total N-SMase activity was increased by 119 ± 41 with time during exponential cell growth, but this increase reached a plateau as cells underwent growth arrest at around days 4–6. Since the activity assay measures all neutral SMase activity (not only nSMase2 activity), it became important to determine whether there were specific increases in nSMase2. Also, we wondered if there was induction of expression of nSMase2 during growth. Therefore, real time PCR measurements of the expression of nSMase2 were conducted, and as shown in Fig. 2B, the levels of mRNA for the endogenous nSMase2 gene were up-regulated ~5-fold when the cells ceased growing compared with exponentially growing cells. These results demonstrate that the expression of nSMase2 mRNA and activity are up-regulated as cells become arrested.

To test whether nSMase2 up-regulation applies to other cell lines, young WI-38 HDF and neuroblastoma SK-N-SH cells were used. Fig. 2, C and D, shows that nSMase2 mRNA was up-regulated by ~3-fold in WI-38 HDF and SK-NHS cells when the cells reached confluence at 7 and 4 days of growth, respectively. These results support the hypothesis that up-regulation of nSMase2 may contribute to the growth arrest induced by confluence.

Down-regulation of nSMase2 Using siRNA—To determine the physiologic role of nSMase2 in cell growth regulation, siRNA was used to down-regulate the levels of nSMase2. The effectiveness and specificity of nSMase2 siRNA were first established by transient overexpression of nSMase2. Cells overexpressing FLAG-tagged mouse nSMase2 (mnSMase2) were transfected with small double-stranded RNA duplexes (mnSMase2 siRNA) specific for mnSMase2 or with control siRNA (SRC) that exhibits no homology to any human DNA sequence based on a BLAST search. As shown in Fig. 3A, anti-FLAG antibodies detected a 75-kDa protein after 6 h of expression, which corresponded to an increase in N-SMase activity of 25-fold (Fig. 3B). Next, nSMase2 protein levels and activity were quantitated after 24 h of transfection with the mnSMase2 siRNA. Fig. 3, A and B, shows that N-SMase activity and protein levels in overexpressing cells incubated in the presence of mnSMase2 siRNA were almost completely abolished by the siRNA. On the other hand, nSMase2 protein expression was unaffected in control siRNA, demonstrating that nSMase2 is a specific target for mnSMase2 siRNA.

Effects of Down-regulation of Endogenous nSMase2 on Cell Cycle—To determine whether the increase in nSMase2 levels plays a role in regulating growth, cell cycle progression was evaluated in MCF7 cells after down-regulation of nSMase2 by siRNA. Since mnSMase2 siRNA was capable of specifically down-regulating overexpressed mouse enzyme (Fig. 3), we used a similar target sequence to down-regulate the endogenous hnSMase2 (hnSMase2RNAi). N-SMase activity in growing and arrested cells was measured after introducing siRNA (day 0), and a control siRNA (166 nM) was used in parallel to test the potential of nonspecific effects. Fig. 4A shows that N-SMase activity after 2 days of growth in cells transfected with the nSMase2 siRNA was similar to the cells treated with the control (SCR) siRNA. These results show that nSMase2 does not contribute much to the total N-SMase activity of the exponentially growing cells. However, the increase in N-SMase activity observed when the cells entered the plateau phase of growth (day 4) was completely annulled by the nSMase2 siRNA. To further confirm those results, nSMase2 mRNA was analyzed by real time RT-PCR. Fig. 4B shows that the siRNA was able to markedly reduce the 4-fold increase of nSMase2 mRNA induced during cell growth arrest. These results establish the ability and the specificity of the siRNA to down-regulate the endogenous nSMase2 protein that was induced during cell growth.
growth. More importantly, the results demonstrate that the induced nSMase2 accounts for nearly all the increase in N-SMase activity in the plateau phase cells but that it probably accounts for very little of the basal activity in growing cells.

The development of the siRNA for nSMase2 allowed us to study the role of the endogenous nSMase2 in the cell cycle using flow cytometric analysis in proliferating and arrested cells. As shown in Fig. 5, in exponentially growing cells treated with the nonspecific siRNA (SCR), 45 ± 3% of the population was in the G0/G1 phase and 40 ± 2% in the S phase of the cell cycle. Instead, at the stationary phase, 71 ± 7.8% of the cell population was at the G0/G1 phase (Fig. 5) and only 22 ± 2% in the S phase. These results are very similar to normally growing untreated cells, demonstrating lack of effect of the nonspecific siRNA.

To determine whether nSMase2 plays a role in the arrested state of the cells at the G0/G1 phase during cell growth, we analyzed the cell cycle in cells preincubated with hnSMase2 siRNA. In proliferating cells transfected with the nSMase2 siRNA, the cell population distribution was similar to the control (Fig. 5, day 2) with 46 ± 2% of the cells in the G0/G1 phase and 43 ± 4% in the S phase of the cell cycle. However, for day 4, treatment with the nSMase2 siRNA decreased the proportion of cells in G0/G1 phase by −32 ± 7.5%, and the cell population in S phase was increased by 59 ± 14%. This cell cycle distribution resembled the one observed in exponentially growing cells. Thus, down-regulation of nSMase2 resulted in a decrease in the cell cycle arrest of cells.

Interestingly, the down-regulation of nSMase2 did not affect cell viability during the exponential phase of growth; however, in arrested cells, the down-regulation of nSMase2 increased the percentage of apoptotic cells from 0.4 to 3.0% (Fig. 5). Similar results were observed by trypan blue assays (data not shown). Taken together, these results not only show that the up-regulation of nSMase2 during growth contributes to the arrest of cells but that it also may protect cells from apoptosis.

**Regulation of Endogenous Ceramide by nSMase2**—Our previous study showed that overexpression of nSMase2 increased ceramide levels (35), and ceramide has been shown repeatedly to induce growth arrest and/or apoptosis, depending on cell type, inducers, and other regulatory factors. Therefore, it became important to determine whether the increase in nSMase2 mRNA at confluence induced the levels of endogenous ceramide. As shown in Fig. 6A, in growth-arrested cells (day 4), the total level of ceramide was increased by 137 ± 31% with respect to the exponentially growing cells (day 2). Thus, endogenous levels of ceramide increase at confluence.

To determine the contribution of nSMase2 to the observed increases in ceramide, nSMase2 was down-regulated with siRNA. As shown in Fig. 6A, down-regulation of nSMase2 did not affect ceramide levels in exponentially growing cells (day 2); however, at the arrested stage (day 4), the increase in ceramide levels significantly decreased (50 ± 16%) by the siRNA. Thus, nSMase2 contributes a major portion of the increase in endogenous ceramide at confluence.

In previous studies, we had shown that activation of the de novo pathway of ceramide synthesis results in the preferential induction of the levels of C18:1-ceramide (41). Therefore, it became of great interest to determine the ceramide molecular species that are induced by nSMase2. To this end, electrospray/MS/MS analysis was performed in control cells and in cells in which nSMase2 was down-regulated. In exponentially growing cells, C16:0, C24:1, and C24:0-ceramides constituted the major ceramide species, and these were not significantly affected by the nSMase2 siRNA (Fig. 6B). However, in confluence-arrested day 4 cells, C16:0, C24:1, and C24:0-ceramides increased 137 ± 15, 370 ± 54, and 266 ± 81%, respectively. To determine whether this increase was due to...
the action of nSMase2, nSMase2 was down-regulated with siRNA. As shown in Fig. 6B, the C24:1 and C24:0 ceramide species were decreased significantly in response to down-regulation of nSMase2 such that most of the increase in these very long chain ceramides was abrogated. Notably, however, there was a further 41%/7.7% increase in C16:0-ceramide in response to treatment with siRNA to nSMase2. These results show that C24:1- and C24:0-ceramides are selectively up-regulated by the increase in nSMase2 activity.

To support this hypothesis, the ceramide species in transient nSMase2 overexpressors were analyzed. As reported previously (35), ceramide levels were increased in nSMase2 overexpressors with respect to the control cells (3.5-fold), and as shown in Fig. 7A, C16:0-, C24:1-, and C24:0-ceramides contributed to the major increase. To determine whether the changes in ceramide species were correlated with changes in sphingomyelin, sphingomyelin species were analyzed in control and nSMase2 overexpressors. Indeed, total sphingomyelin levels were decreased by 29%/14% in nSMase2 overexpressors, and C24:1- and C24:0-sphingomyelins contributed approximately to 60% of this decrease (Fig. 7B). Taken together, these results support a role of nSMase2 in regulating very long chain ceramides during cell growth.

**Downstream Targets for nSMase2—**Because pRb is a critical regulator of the G1-S boundary of the cell cycle, and since we had previously shown that exogenous ceramide induces pRb
dephosphorylation (activation), we next attempted to determine the effect of confluence on the state of pRb phosphorylation and the role of nSMase2 in this process. As shown in Fig. 8A, pRb was decreased, and a higher proportion was in the dephosphorylated state when the cells became arrested (day 4). Analysis of the Western blot by densitometry (Fig. 8B) indicated that the level of hypophosphorylated pRb was increased −6-fold at day 4 of growth, in correlation with the increase in ceramide levels. To establish whether nSMase2 plays a role in regulating pRb, nSMase2 siRNA was used to specifically downregulate nSMase2. Indeed, nSMase2 siRNA was able to revert significantly the decrease in the levels and phosphorylation state of pRb (Fig. 8, A and B). These results establish a role for nSMase2 in the regulation of pRb phosphorylation.

Another related mechanism that could be involved in confluence arrest involves the inhibition of cyclin-dependent kinases (CDKs). It has been shown that exogenous ceramide enhances the expression of the protein p21WAF1, one of the key inhibitors of CDKs. To determine whether p21WAF1 may be regulated by up-regulation of nSMase2 during growth, total cellular proteins from exponentially growing and arrested cells were prepared, and p21WAF1 protein levels were analyzed by Western blotting. Cells growing at the exponential phase revealed low basal levels of p21WAF1 protein (Fig. 8, C and D); however, when the cells were arrested, an increase in p21WAF1 expression was observed (289 ± 47%). Fig. 8, C and D, shows that down-regulation of nSMase2 with siRNA resulted in a significant decrease in p21WAF1 expression, indicating a role for nSMase2 in the induction of p21. These results on pRb and p21WAF1 provide specific links between nSMase2 and specific downstream targets.

**nSMase2 Is Translocated to the Plasma Membrane in Response to High Cell Density**—Given the above results, it became important to determine the subcellular localization of nSMase2 and whether this is regulated during cell growth.

Immunofluorescence studies where done with an nSMase2 3′-GFP tag (Fig. 9, A–C) or FLAG tag (Fig. 9, D–F), and the results show that nSMase2 was located at the intercellular junction in subconfluent cells, but also a significant amount was distributed throughout the cell (Fig. 9, A, B, D, and E). In contrast, in confluent cells, nSMase2 became located mainly at the cell periphery (Fig. 9, C and F). These results show specific intracellular cytosolic localization of nSMase2 with preference to locate at intercellular junctions such that at high density the enzyme becomes predominantly associated with the intercellular borders.

**DISCUSSION**

Several reports have shown that neutral sphingomyelinases are regulated in response to cytokines and stress stimuli, and they have suggested that these enzymes may be have important roles in the generation of ceramide in response to these stimuli and in mediating some of their actions on apoptosis and/or cell cycle arrest (42, 43). However, the lack of molecular identification of these enzymes and the absence of specific tools to modulate their activity have significantly handicapped efforts to delineate upstream and downstream targets of specific pathways involving neutral SMases (3, 44).

In a previous report, we showed that nSMase2 functions as a bona fide SMase and that overexpression of nSMase2 suppresses cell growth (35). nSMase2 was previously isolated as a confluent 3Y1 cell-associated gene (cca1) whose corresponding mRNA was up-regulated in growth-arrested confluent but not in growing subconfluent 3Y1 cells. Interestingly, when the cDNA was introduced to a derivative cell line of 3Y1 (3Y1 BU), which continue to grow without changing their morphological characteristics after reaching confluence, a restoration of the confluent 3Y1-type growth pattern was observed (36). In this study, we aimed at determining the role of the endogenous nSMase2 in confluence-induced ceramide generation.

*Fig. 6.* Ceramide levels and molecular species during cell growth: Effect of nSMase2 siRNA. MCF7 cells treated with scrambled (SCR) siRNA or hnSMase2 siRNA were collected at the indicated time points and assayed for total endogenous ceramide levels (A) and ceramide species (B) as described under “Experimental Procedures.” The ceramide values were normalized to total lipid phosphate. Results are average ± S.D. of three different experiments. Statistical significance was calculated with respect to scrambled siRNA at 2 days (*, p < 0.01; **, p < 0.001; ††, p < 0.01; †††, p < 0.05) and scrambled siRNA at 4 days (†, p < 0.01; ††, p < 0.05).
ceramide composition in MCF7 overexpressing the mouse nSMase2 showed that, similar to the endogenous human nSMase2, the enzyme primarily induced the levels of very long chain ceramides, which correlated with a decrease in \( C_{24:0} \) and \( C_{24:1} \)-sphingomyelins.

It is also interesting to note that, parallel to the decrease of very long chain ceramides, down-regulation of nSMase2 induced an increase in long chain ceramide (\( C_{16:0} \)). These results suggest that nSMase2 may negatively regulate the levels of \( C_{16} \)-ceramide, probably through feedback. More studies are required to discern these mechanisms.

The increase in \( C_{16:0} \)-ceramide during nSMase2 down-regulation is consistent with an increase in the percentage of apoptotic cells (41, 45). Other studies have shown specific generation of \( C_{16} \)-ceramide in response to Fas- and ionizing radiation-induced apoptosis (45). Indeed, \( C_{16} \)-ceramide was recently identified as the predominant ceramide that is up-regulated in Ramos B cells during the initial phase of apoptosis induced by activation of the B cell receptor, preceding the onset of apoptosis (46). Evidence was provided in that study to show that \( C_{16} \)-ceramide was specifically derived from the \textit{de novo} pathway and that inhibition of formation of this ceramide prevented B cell receptor-induced cell death upstream of the mitochondrial pathway.

It is also becoming clear that the same stimulus may activate more than one pathway of ceramide generation. For example, treatment of MCF7 cells with TNF-\( \alpha \) resulted in a combined response involving ceramide derived from both SMase activity and \textit{de novo} pathway (47), although the specific molecular species were not identified. In Ramos cells, it was shown that B cell receptor cross-linking, which caused early elevation of \( C_{16} \)-ceramide, resulted in the late formation of very long chain ceramides, downstream of the mitochondrial pathway (46). No specific role for that ceramide was defined. Our results clearly demonstrate that different ceramide species are formed during cell growth and may have different effects in the regulation of cell cycle and apoptosis. Thus, these studies are beginning to characterize specific pathways of ceramide formation involving specific molecular species.

Results from this study also provide insight into specific mechanisms that begin to couple nSMase2-generated ceramide to cell cycle arrest. Both pRb and p21\textsuperscript{WAF1} function as key regulators of the cell cycle, and evidence has been provided for the ability of ceramide to regulate both pRb and p21\textsuperscript{WAF1} (6, 13, 21, 22).

In the present study, we show that nSMase2 activation induces hypophosphorylation of pRb and induction of p21\textsuperscript{WAF1} levels and that down-regulation of nSMase2 interferes with the effects of confluence-induced growth arrest on p21 and pRb phosphorylation. Therefore, these results establish pRb and p21 as the first putative downstream targets for nSMase2 in its regulation of the cell cycle.

Interestingly, the results from this study show that the regulation of nSMase2 during cell growth occurs at least at two levels, at the level of RNA expression and by cellular relocalization. Several proteins that are regulated by cell density have been shown to have different localization depending on the cell density, including \( \beta \)-catenins (48), cadherins (49–52), and nitric oxide synthase (53). Hofmann \textit{et al.} (33) had shown that endogenous nSMase2 displayed an intracellular localization, and little signal was observed at the plasma membrane (33). The current study shows that nSMase2 in subconfluent cells was primarily intracellular, and the localization at the plasma membrane was enriched/restricted to the regions of cell-cell contact interactions. However, at high cell density, nSMase2 was mainly detected at...
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The plasma membrane with loss of enzyme from intracellular sites, suggesting that the localization of nSMase2 is a dynamic process that may be subjected to a regulatory mechanism. These findings raise interesting questions as to the role and the mechanisms that are involved in this translocation. Interestingly, the carboxyl-terminal region of nSMase2 harbors several motifs that may play a role in its localization. For example, the tyrosine-containing sequences [529THY532] and [581YAF584] fit the consensus sequence YXXH (where X represents any amino acid and H is an amino acid with a bulky hydrophobic side chain (Leu, Ile, Val, Met, or Phe)) that serves mainly as a signal of internalization from the plasma membrane (54–57). Another interesting motif is the acidic cluster [558YDED561] separated by 5 amino acids from a dileucine motif. This motif has been shown to play a role in the endosomal sequestration of several proteins such as the insulin-responsive aminopeptidase, the proprotein convertase PC6B, and GLUT-4 (58–60). Further studies will address the role of these motifs in nSMase2 localization.

The ability of nSMase2 to regulate cell growth leads to the prediction that loss of nSMase2 expression could contribute to proliferation of cancer cells and/or to their invasiveness. Interestingly, nSMase2 is localized to chromosome 16q22.1, and genetic alterations in this region have been implicated in the progression of different cancers (61–63). Eight members of the cadherin family have been also mapped to the long arm of the chromosome 16, and six were localized within the nSMase2-containing cluster at 16q21–22.1 (64). Cadherins are involved in contact inhibition and adhesion (65, 66), and reduced levels of cadherin expression have been associated with tumor progression (67–69). The close chromosomal localization of cadherins and nSMase2 and their conserved order in human and mouse chromosomes might indicate that they share genetic control elements. Supporting this observation, members of this family of cadherins are regulated by cell density and confluence (32, 50, 51).

In conclusion, this study demonstrates a significant induction of nSMase2 during cell-cell contact inhibition and a role for this specific N-SMase in cell cycle regulation and ceramide formation. Further elucidation of the mechanisms by which cell growth and cell-cell contact regulate nSMase2 expression and localization to the plasma membrane and the mechanisms of action of the very long chain ceramides are essential to delineate this emerging role of nSMase2 as a growth suppressor.

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**Fig. 8. Effects of nSMase2 siRNA on pRb activation and p21WAF1 expression during growth arrest.** Cells treated with scrambled (SCR) siRNA (lanes 1 and 3) or hnSMase2 siRNA (lanes 2 and 4) were collected at day 2 (lanes 1 and 2) or day 4 of growth (lanes 3 and 4). Whole cell lysates were normalized to total protein, and 100 µg was separated by SDS-PAGE. Total pRb and p21WAF1 expression levels were analyzed by Western blot (A and C) and quantified by densitometry (B and D). The data were normalized for total pRb (B) or total protein loaded (D) and were plotted as -fold increase with respect to the scrambled control at day 2. Equal loading was verified by using anti-β-tubulin (A) and β-actin (C) antibodies. The results shown in A and C are representative of at least three independent experiments. Error bars, the average ± S.D. of a least three independent experiments in B and D. Statistical significance was calculated with respect to scrambled control day 4 (*, p < 0.05).
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