The Geranylgeranyltransferase I Inhibitor GGTI-298 Induces Hypophosphorylation of Retinoblastoma and Partner Switching of Cyclin-dependent Kinase Inhibitors

A POTENTIAL MECHANISM FOR GGTI-298 ANTITUMOR ACTIVITY

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The geranylgeranyltransferase I inhibitor GGTI-298 has recently been shown to arrest human tumor cells in the G1 phase of the cell cycle, induce apoptosis, and inhibit tumor growth in nude mice. In the present manuscript, we provide a possible mechanism by which GGTI-298 mediates its tumor growth arrest. Treatment of the human lung carcinoma cell line Calu-1 with GGTI-298 results in inhibition of the phosphorylation of retinoblastoma protein, a critical step for G1/S transition. The kinase activities of two G1/S cyclin-dependent kinases, CDK2 and CDK4, are inhibited in Calu-1 cells treated with GGTI-298. Furthermore, GGTI-298 has little effect on the expression levels of CDK2, CDK4, CDK6, cyclins D1 and E, but decreases the levels of cyclin A. GGTI-298 increases the levels of the cyclin-dependent kinase inhibitors p21 and p15 and had little effect on those of p27 and p16. Most interesting is the ability of GGTI-298 to induce partner switching for several CDK inhibitors. GGTI-298 promotes binding of p21 and p27 to CDK2 while decreasing their binding to CDK6. Reversal of partner switching and G1 block was observed after removal of GGTI-298. Furthermore, GGTI-298 treatment results in an increased binding of p15 to CDK4, which is paralleled with decreased binding to p27. The results demonstrate that the GGTI-298-mediated G1 block in Calu-1 cells involves increased expression and partner switching of CDK inhibitors resulting in inhibition of CDK2 and CDK4, and retinoblastoma protein phosphorylation.

Protein prenylation is an important posttranslational modification that is required for cellular localization and biological function of many proteins (1). These covalent attachments of farnesyl (C15) or geranylgeranyl (C20) to cysteines at the carboxyl-terminal with GGTase I preferring leucine or isoleucine and FTase preferring Met or Ser in the X position (1). FTase, farnesyltransferase II, on the other hand, geranylgeranylates on two cysteines in proteins that end in XXCC, CCX, or CXC sequences. Many prenylated proteins are small G-proteins that are integral components of proliferative signal transduction pathways (1). For example, Ras farnesylation is required for its ability to transduce growth signals from receptor tyrosine kinases to transcription factors and the cell cycle machinery that regulates cell division (2). Furthermore, mutated Ras is found in about 30% of all human cancers and is believed to cause malignant transformation by constitutive activation of abnormal growth (3). Farnesylation of oncogenic Ras is required for its cancer-causing activity (4). Similarly, the Rho family of small G-proteins such as RhoA and Rac1 require geranylgeranylation for their biological function. One of their key biological roles is to allow cells to traverse the G1 phase of the cell cycle and begin DNA synthesis in S phase (5). Recently, RhoA and Rac1 have been shown to be required for malignant transformation by Ras (6, 7). Furthermore, constitutively activated RhoA and Rac1 can also lead to oncogenic transformation (6, 7). The overwhelming evidence implicating the Ras and Rho family of proteins in aberrant proliferative pathological conditions such as cancer and cardiovascular diseases prompted us and others (reviewed in Ref. 8) to design and synthesize FTase and GGTase I inhibitors.

FTI-277 and GGTI-298 are CAAX peptidomimetics that potently and selectively inhibit FTase and GGTase I, respectively (9, 10). We have found FTI-276 and its methyl ester FTI-277 to be potent inhibitors of oncogenic Ras processing and signaling (9). FTI-276 also potently inhibited the growth in nude mice of human tumors with multiple genetic alterations such as K-Ras mutation and p53 deletion (11, 12). FTIs from several other groups have also shown potent antitumor efficacy without toxicity in several animal models (8). Furthermore, several FTIs are presently in phase I clinical trials (13).

Selective inhibition of protein geranylgeranylation with GGTI-298 has major consequences on several biological pathways. Pretreatment of fibroblasts with GGTI-298, blocks PDGF- and epidermal growth factor-dependent tyrosine phosphorylation of their corresponding tyrosine kinase receptors (14). In contrast, selective inhibition of protein farnesylation has no effect on receptor tyrosine kinase phosphorylation (14). Furthermore, GGTI-298 inhibits the growth in nude mice of PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis.

6930 This paper is available on line at http://www.jbc.org
human tumors by a mechanism that is not yet known (12). One possible mechanism may involve GGTI-298-mediated G1 phase block and apoptosis in cultured human tumor cells (15). The ability of GGTI-298 to inhibit proliferation is not limited to human tumor cells that are of epithelial and fibroblast origin. GGTI-298 also has a major effect on the proliferative pathways of smooth muscle cells (16). For example, GGTI-298 is very effective at inducing G1 arrest and apoptosis in rat pulmonary artery smooth muscle cells. Furthermore, GGTI-298 enhances the ability of interleukin-1β to induce nitric oxide synthase-2 in the same cells (17). This induction of nitric oxide synthase-2 results in a large increase in the production of the nitric oxide radical, which is known to be inhibitory to smooth muscle cell proliferation. GGTI-298 also inhibited the ability of PDGF, interleukin-1β, and activated Ras to induce superoxide production in smooth muscle cells (18). Taken together, the data indicate that GGTI-298 has antiproliferative effects on fibroblasts, epithelial, and smooth muscle cells, and this cell growth inhibition appears to be mediated through a G1 phase arrest. Recently, we have shown that in human tumors this G1 arrest correlated with an induction of a cyclin-dependent kinase inhibitor p21WAF1 (19). However, whether this induction of p21WAF1 is responsible for the G1 arrest is not yet known.

The G1 to S phase transition of the mammalian cell division cycle is a highly regulated step. The key regulators of G1/S progression are a series of kinases that depend on cyclins for activation (for review, see Refs. 20–22). For example, the α-type cyclins are made in early G1, bind to and activate the G1 cyclin-dependent kinases CDK4 and CDK6. Activation of these kinases also requires phosphorylation by CDK kinases such as Cdk4 or cyclin H-CDK7 and dephosphorylation by phosphatases such as cdc25 (20). At this stage, the cell has reached a critical checkpoint of its cell cycle called the restriction point (R) at which time the cell checks that all is ready for DNA synthesis. Cyclin D-CDK4 and cyclin D-CDK6 complexes are now active and phosphorylate the retinoblastoma gene product pRb. Phosphorylated pRb dissociates from the transcription factor E2F, which, once freed from pRb, is able to induce the expression of several genes that prepare the cell for DNA synthesis (20). Among these is cyclin E, which activates CDK2, a kinase required for late G1 to early S phase transition. The cyclin E-CDK2 complex hyperphosphorylates pRb, and the cell proceeds into S phase. At this time cyclin A expression is high, whereas cyclins D and E have been degraded (20). The cyclin A-CDK2 complex maintains the phosphorylation of pRb to sustain DNA replication. Finally CDK kinase activities are highly regulated by two families of CDK inhibitors, CKIs such as p21 and p27 and the INKs such as p15 and p16 (20–22). These inhibitors play a key role in making sure the cells stop at the R point if any DNA damage is detected. This allows the cells to repair the damage before replicating their DNA. Growth factors such as PDGF and epidermal growth factor activate several pathways, some of which have been shown to directly regulate the cell cycle (22). For example, activation of the Ras/Raf/MEK/ERK kinase cascade, which results in increased expression of cyclin D1 that in turn will activate CDK4/CDK6 allowing cells to traverse the R point. PDGF activation of the Ras/RhoA pathway results in the degradation of p27, which also will have the same overall effect of allowing cells to traverse the R point and enter S phase of the cell cycle (23).

The G1 arrest brought about by GGTI-298 inhibition of protein geranylgeranylation could be due to effects on several important steps in G1/S such as the inhibition of expression of cyclins D and/or E, CDK2, CDK4 and/or CDK6, or increased expression of CKIs and/or INKs. The work described in this study suggests a possible mechanism for GGTI-298 growth arrest involving increased expression and partner switching of CDK inhibitors, resulting in inhibition of CDK2 and CDK4, and pRb phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Synthesis of CAAX Peptidomimetic**—The GGTase I-specific peptidomimetic GGTI-298 was synthesized as described previously (10, 14). Cells and Culture—Human tumor cell lines Calu-1 and A-549 (lung carcinomas), T-24 (bladder carcinoma), and A-253 (head and neck squamous cell carcinoma) were purchased from ATCC (Manassas, VA) and grown in McCoy’s 5A (Calu-1, T-24, and A-253) and F12K (A-549) media at 37 °C in a humidified incubator containing 10% CO2.

**Western Blotting**—Cells were treated with GGTI-298 (15 μM) for 48 h, harvested, and lysed in HEPES lysis buffer as described previously (11, 14). Proteins were then resolved by 12.5% or 7% SDS-PAGE gel and immunoblotted with antibodies against Rap1A/Krev-1(1-121), p21WAF1 (C-190), cyclin E (C-19), cyclin D1 (72–13G), CDK2 (M2), CDK4 (H-22), CDK6 (C-21), p16INK4A (C-20), p15INK4B (C-20), cyclin A (H-432) (all from Santa Cruz Biotechnology, Santa Cruz, CA), p27KIP1 (G173–524), and pRb (G3–245) (from Pharmingen, San Diego, CA). The ECL blotting system (NEF Life Science Products) was used for detection of positive antibody reactions (14).

**Flow Cytometry Analysis**—Cells were treated and harvested as described for Western blotting, and nuclei were stained with propidium iodide. DNA content was analyzed by fluorescence-activated cell sorter as described previously (19).

**Immunoprecipitations**—Cells were treated and harvested as described above for Western blotting. Lysates (500 μg) were then immunoprecipitated with polyclonal antibodies to CDK2 (M2), CDK4 (H-22), and CDK6 (C-21). The immunoprecipitates were then electrophoresed on a 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with p15, p16, p21, and p27 as described above.

**Cyclin-dependent Kinase Assays**—To measure the activity of CDK2, histone H1 was used as the substrate; for CDK4 and CDK6, GST-Rb (C-terminal fragment of pRb) was used as a substrate. The CDK immunoprecipitates were resuspended in 10 μl of 50 mm Hepes (pH 7.4) containing 10 mm MgCl2, 5 mm MnCl2, 1 mm dithiothreitol, 10 μCi [γ-32P]ATP and 100 μg/ml histone H1 (BM) or 20 μg/ml GST-Rb, and then incubated for 30 min at 30 °C with occasional mixing. The reaction was terminated with an equal volume of 2× loading buffer (93.75 mm Tris, pH 6.8, 15% glycerol, 3% SDS, 7.5% b-mercaptoethanol). The sample was fractionated by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography.

**RESULTS**

GGTI-298 Induces Accumulation of Hypophosphorylated pRb in the Human Lung Carcinoma Calu-1—GGTI-298 was previously shown to inhibit the growth in nude mice and to induce G1 block of human tumor cells (12, 15). We sought to understand how GGTI-298 prevents Calu-1 cells from traversing G1 and entering S phase of the cell cycle. We first determined the ability of GGTI-298 to affect phosphorylation of pRb, one of the key events required for G1/S transition. Calu-1 cells were treated for 48 h with GGTI-298 (15 μM), and the cell lysates were immunoblotted with an anti-pRb antibody that recognizes both hypo- and hyperphosphorylated forms of pRb as described under “Experimental Procedures.” Other cell lysate aliquots were immunoblotted with antibodies to either Rap1A or RhoA, small G-proteins that are exclusively geranylgeranylated. Finally, a set of cells were analyzed by flow cytometry to determine the proportion of cells at different phases of the cell cycle. Fig. 1A shows that Calu-1 cells treated with the vehicle contained predominantly hyperphosphorylated pRb. Treatment with GGTI-298 resulted in hypophosphorylation of pRb (Fig. 1A). Hypophosphorylation of pRb correlated with inhibition of protein geranylgeranylation of the GGTase I substrates, Rap1A and RhoA, and increased the proportion of Calu-1 cells in the G1 phase of the cell cycle (Fig. 1A). Because processed and unprocessed RhoA migrated closely (Fig. 1A), we confirmed the effects of GGTI-298 on the processing of RhoA by isolating membranes and cytosolic fractions and showing that GGTI-298 decreases the membrane levels of RhoA while inducing accumulation of RhoA in the cytosol (Fig. 1B).
GGTI-298 Inhibits the Kinase Activities of CDK2 and CDK4—We next evaluated the ability of GGTI-298 to inhibit G1 phase cyclin-dependent kinases that phosphorylate pRb. Calu-1 cells were treated with GGTI-298 for 48 h, and the lysates immunoprecipitated with anti-CDK2, anti-CDK4, or anti-CDK6 antibody as described under “Experimental Procedures.” Fig. 2 shows that CDK2, CDK4, and CDK6 from control Calu-1 cells were active and phosphorylated histone H1 (CDK2) and GST-Rb (CDK4 and CDK6) in vitro. Treatment with GGTI-298 blocked the activity of CDK2, inhibited CDK4 and CDK6 activities by 75% and 30%, respectively (Fig. 2).

Effects of GGTI-298 on the Expression of Various Cell Cycle Components—The mechanism by which GGTI-298 results in inhibition of the activities of CDKs could involve inhibition of the expression of the G1 phase cyclin-dependent kinases that phosphorylate pRb. Calu-1 cells were treated with GGTI-298 for 48 h, and the lysates immunoprecipitated with anti-CDK2, anti-CDK4, or anti-CDK6 antibody as described under “Experimental Procedures.” Fig. 2 shows that CDK2, CDK4, and CDK6 from control Calu-1 cells were active and phosphorylated histone H1 (CDK2) and GST-Rb (CDK4 and CDK6) in vitro. Treatment with GGTI-298 blocked the activity of CDK2, inhibited CDK4 and CDK6 activities by 75% and 30%, respectively (Fig. 2).

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GGTI-298 treatment induced pRb hypophosphorylation. A. Calu-1 cells were treated for 48 h with GGTI-298 (15 μM), cell lysates were prepared, separated by SDS-PAGE, and immunoblotted with either an anti-pRb1, anti-RhoA, or anti-Rap1A antibody as described under “Experimental Procedures.” U and P designate unprocessed and processed forms of Rap1A. The proportion of Calu-1 cells in G1 was determined by flow cytometry as described under “Experimental Procedures.” B. Calu-1 cells were treated as described in A, except that membrane (Mem) and cytosolic (cyto) fractions were isolated before SDS-PAGE immunoblotting with anti-RhoA antibody. Data are representative of five independent experiments except for RhoA (two independent experiments).
also induced an increased association of CDK4 with p15 while decreasing its association with p27.

We next determined whether the GGTI-298-induced partner switching is unique to Calu-1 cells. To this end, we treated three other human tumor cell lines; A-549 lung carcinoma, T-24 bladder carcinoma, and A-253 head and neck squamous cell carcinoma with GGTI-298, immunoprecipitated the lysates with CDK2 and CDK6 antibodies, and blotted with p27 antibody as described under “Experimental Procedures.” Fig. 5 shows that in all three cell lines treatment with GGTI-298 increased the levels of p27 associated with CDK2 while it decreased the levels associated with CDK6.

**DISCUSSION**

Although there are more geranylgeranylated proteins than farnesylated proteins, more efforts have been spent on designing, synthesizing, and biologically characterizing FTase rather than GGTase I inhibitors (8). The intense search for FTase inhibitors was prompted some years ago by the realization that farnesylation is required for the cancer-causing activity of the important oncoprotein Ras (8). Recently, however, more attention has been directed toward understanding the effects of inhibiting (by GGTase I inhibitors) the function of geranylgeranylated proteins. This is primarily due to the discovery that some geranylgeranylated proteins such as those from the Rho family are essential for normal and aberrant proliferation in several cell types (1, 5). In addition to their ability to inhibit human tumor growth, GGTase I inhibitors may also have great therapeutic potential in cardiovascular diseases. For example, GGTI-298 blocks the ability of smooth muscle cells to proliferate by inducing a G1 block and apoptosis (16). This may be related to the inhibition by GGTI-298 of PDGF and Ras induction of superoxide formation (18) suggesting that a geranylgeranylated protein downstream of Ras is critical to events regulating cell division. This is consistent with a recent report, which shows that the geranylgeranylated protein Rac1 mediates superoxide formation and transformation by Ras (24). In this study, we demonstrate that GGTI-298 treatment of the human lung carcinoma Calu-1 cells results in a large increase of the CDK inhibitor p21 and a modest increase of p15 with little effect on p27 and p16. Upon treatment with GGTI-298, p21 and p27 switched partners from CDK6 to CDK2, whereas p15 became bound to CDK4. The
effects of GGTI-298 on the observed partner switching was reversible. We found that removal of GGTI-298 resulted in reversal of the G₁ phase block, which was paralleled by a reversal of partner switching of p27 from CDK6 to CDK6.

The fact that in dividing cells, CDKs are bound to some inhibitors is not surprising, because low levels of such inhibitors may be required to stabilize cyclins with the corresponding CDKs (25). This is also consistent with the suggestion that some CDKs may serve as cellular reservoirs for low levels of inhibitors (26). Furthermore, although there was little increase in total cellular levels of p27 after GGTI-298 treatment, the amount of p27 bound to CDK2 was increased. This suggests that GGTI-298 induced partner switching for p21 and p27 from CDK4, bound to CDK2. Taken altogether, the data suggests that GGTI-298 induced partner switching for p21 and p27 that was released from CDK6, and to a lesser degree amount of p27 bound to CDK2 was increased. This suggests in total cellular levels of p27 after GGTI-298 treatment, the inhibitors (26). Furthermore, although there was little increase some CDKs may serve as cellular reservoirs for low levels of CDKs (25). This is also consistent with the suggestion that tors may be required to stabilize cyclins with the corresponding inhibitors is not surprising, because low levels of such inhibi-

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suggested that GGTI-298 induction of p21

Jun kinase by Rac1. It would be of great interest to determine

affect genes that are regulated by AP-1. Furthermore, very

tion by activating the Jun kinase pathway that in turn will

genic Ras transformation (6, 7). Rac1 may regulate transcrip-

into G1-arrested cells, promote progression to S phase (5). Fur-

inhibition of RhoA geranylgeranylation (29). Furthermore, it

reversal of partner switching of p27 from CDK2 to CDK6.

The fact that inhibition of protein geranylgeranylation re-

results in G1 arrest suggests that some geranylgeranylated pro-

teins are required for cell progression from G₁ to S. The most

likely candidates are members of the Rho family of proteins

such as RhoA and Rac1. In this study, we showed that GGTI-

298 inhibits the processing of RhoA, and recently we have

suggested that RhoA represses p21\textsuperscript{ind} transcription and suggested that GGTI-298 induction of p21\textsuperscript{ind} is mediated by inhibition of RhoA geranylgeranylation (29). Furthermore, it has been shown that both RhoA and Rac1, when microinjected into G₁-arrested cells, promote progression to S phase (5). Fur-

thermore, dominant-negative Rac1 and RhoA reverse onco-

genic Ras transformation (6, 7). Rac1 may regulate transcription by activating the Jun kinase pathway that in turn will affect genes that are regulated by AP-1. Furthermore, very recently Tapon et al. (28) discovered a novel effector of Rac1, POSH, a kinase that appears to be required for activation of Jun kinase by Rac1. It would be of great interest to determine whether activated Jun kinase or POSH would rescue the cells from the GGTI-298 mediated G₁ block. These studies, which are in progress, will further enhance our understanding of how inhibition of protein geranylgeranylation results in G₁ arrest.

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