J. Biochem. 120, 1118-1122 (1996)

Inhibition of G1 Cyclin Expression in Normal Rat Kidney Cells by Inostamycin, a Phosphatidylinositol Synthesis Inhibitor[1]

Atsuko Deguchi, Masaya Imoto,[2] and Kazuo Umezawa

Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223

Received for publication, July 3, 1996

We previously reported that inostamycin, an inhibitor of CDP-DG:inositol transferase, inhibited cell proliferation in normal rat kidney (NRK) cells by blocking cell cycle progression at the G_1 phase. In the present paper, we report the effect of inostamycin on the serum-induced activation of Ser/Thr protein kinases that are involved in G_1 progression. In quiescent NRK cells mitogen-activated protein kinase (MAP kinase) and casein kinase II were activated within 15 min after serum addition. Neither activation was affected by the treatment with inostamycin. However, in the inostamycin-treated cell, cyclin-dependent kinase 2 (CDK2) failed to be activated after serum stimulation. Since serum-induced expression of cyclin E was also suppressed by inostamycin, this inhibitor would appear to block CDK2 activation by inhibiting cyclin E expression. Furthermore, inostamycin also inhibited cyclin D1 expression induced by serum; and consequently, hyperphosphorylation of retinoblastoma protein (pRB) by RB-kinases such as CDK4 and CDK2 was abolished, which would result in elimination of functional inactivation of pRB. Thus, early G_1 arrest in NRK cells by inostamycin is due to the inhibition of cyclin D1 and E expressions.

Key words: cdk2, cyclin D, cyclin E, inostamycin, phosphatidylinositol.

Mitogenic stimulation of quiescent cells induces an array of biochemical events that culminate in DNA synthesis and cell division. The process is initiated at the cell surface by the activation of receptor tyrosine kinases and G proteins, and is propagated intracellularly by multiple Ser/Thr protein kinases. Of the latter, the most essential enzymes are members of the mitogen-activated protein kinase (MAP kinase) family. MAP kinases mediate the phosphorylation and activation of nuclear transcription factors that regulate cell growth. The activity of these kinases is acutely stimulated by virtually every mitogenic stimulus, including growth factor, T cell antigens, and phorbol esters. Therefore, MAP kinase is likely to represent a site of integration for common signaling mechanisms in cell growth (for review see Refs. *1-3*). Furthermore, receptor tyrosine kinase-mediated MAP kinase activation as well as G protein-mediated MAP kinase activation involves a series of SH2- and SH3-dependent protein-protein interactions between Shc, Grb2, and Sos, resulting in Ras activation (*4-9*).

Casein kinase II (CKII) is a ubiquitous Ser/Thr protein kinase located in the cytosol as well as in the nucleus of eukaryotic cells (*10*). The physiological activity of CKII is transiently stimulated by a number of polypeptide hormones or serum (*11-14*). CKII may play an especially important role in the regulation of nuclear proteins, because it phosphorylates nuclear proteins including Myc, Fos, Myb, and p53 (*15*). Moreover, cell-cycle transition from G_0 to S phase requires the presence of a certain functional level of CKII (*16, 17*).

The eukaryotic cell-cycle progression is controlled by the sequential activation of cyclin-dependent kinases (CDKs). They are inactive alone, but are activated by association with cyclins (*18*). Three cyclins, named cyclins C, D, and E, are expressed during the G_1 phase in mammalian cells. Cyclin C is reported to associate with CDK8 (*19*), but the precise function of cyclin C/CDK8 is obscure. Cyclin D associates with several kinases, namely, CDK2, CDK4, CDK5, and CDK6 (*20, 21*). Expression of cyclin D depends on the presence of growth factors and may be a signal that nutrient levels are sufficient for cell division (*22*). Cyclin E associates with CDK2 to form an active kinase (*23, 24*). The cyclin E concentration peaks at the G_1 to S transition, suggesting a role of cyclin E in the initiation of DNA synthesis. Moreover, complete activation of CDKs also requires phosphorylation of their conserved threonine residue by a CDK-activating kinase (CAK) (*25*). CAK itself is a CDK complex consisting of a catalytic subunit, CDK7/MO15, and a regulatory subunit, cyclin H (*26-28*). Furthermore, CAK associated with MAT1 (*29*). Activated CDK4/cyclin D complex as well as CDK2/cyclin E can phosphorylate the retinoblastoma protein (pRB) (*30*). Phosphorylation of the pRB and the release of RB-associated protein such as E2F is correlated with the transition across the G_1 checkpoint (*31*). Then, free E2F is available to transcriptionally active genes encoding proteins critical for S-phase functions, including deoxynucleotide biosynthesis (*32*).

Previously, we isolated a novel polyether compound, inostamycin, from *Streptomyces* as an inhibitor of CDP-

[1] This work was partly supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-45-563-1141, Fax: +81-45-562-7625

DG:inositol transferase (*33*). The physiological role of CDP-DG:inositol transferase is still obscure. However, we also found that *src*- and *erbB2*-transformed cells have a higher level of this enzyme activity than normal cells and that this enzyme activity is regulated by tyrosine kinase (*34*). In quiescent normal rat kidney (NRK) cells, phosphatidylinositol (PI) synthesis catalyzed by CDP-DG:inositol transferase is stimulated by the addition of mitogenic growth factors or serum. Therefore, PI synthesis is considered to be involved in the regulation of cell growth. Indeed, inhibition of PI synthesis by inostamycin blocks the cell cycle progression at the G_1 phase in NRK cells (*35*). However, the mechanism of growth arrest induced by inostamycin has remained unclear. In the present study, therefore, we examined the effect of inostamycin on the serum-mediated activation of Ser/Thr protein kinases leading to G_1 progression. We found that the serum-induced activation of CDK2 was abrogated by the impaired expression of G_1 cyclins in inostamycin-treated NRK cells.

MATERIALS AND METHODS

Materials—Inostamycin was isolated from *Streptomyces* as described previously (*33*). NRK-49F cell line (ATCC CRL-1570) was obtained through Flow Laboratories. Heparin and α-casein were purchased from Sigma; and anti-CDK2, anti-cyclin D, anti-cyclin E, and anti-MAP kinase (Erk2), from Upstate Biotechnology. Histone H1 was obtained from Boehringer-Mannheim; anti-pRB (PMG-245), from Pharmingen, and [γ-^{32}P]ATP, from New England Nuclear.

Cell Synchronization—NRK cells were cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (CS) and then for 72 h in medium containing 0.2% CS. Thereafter, the cells were arrested at G_0 phase. Quiescent NRK cells were stimulated by addition of 5% CS.

Western Blotting Analysis for MAP Kinase—NRK cells were harvested with Dulbecco's phosphate-buffered saline (PBS) and lysed in MAP kinase lysis buffer (20 mM Tris-HCl [pH 8.0], 1% NP-40, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 400 μM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml antipain) for 1 h at 4°C. Insoluble material was removed by centifugation at 10,000 × *g* for 15 min at 4°C. The lysates were electrophoresed on 10% polyacrylamide gel and then electrophoretically transferred to a PVDF membrane. The filter was blocked with 6% CS, washed, and incubated with a solution containing primary antibody. After having been washed, the filter was incubated with biotin-conjugated secondary antibody (Amersham), washed, and then incubated with streptavidin-alkaline phosphatase conjugate reagent (Amersham). Finally, the protein bands were detected with NBT and BCIP (Gibco BRL).

CKII Kinase Assay—NRK cells were harvested with PBS and homogenized in homogenizing buffer (20 mM Tris-HCl [pH 7.6], 60 mM β-glycerophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 0.1 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 100 μg/ml leupeptin). The supernatant obtained by centifugation at 15,000 × *g* for 15 min was used as the enzyme source. The enzyme preparation was reacted with α-casein and [γ-^{32}P]ATP, with or without heparin, for 15 min at 30°C. The reaction was terminated by addition of the same volume of 2 × loading buffer (42 mM Tris-HCl [pH 6.8], 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, 0.002% bromophenol blue), and the mixture was boiled for 5 min. The samples were electrophoresed on 12.5% polyacrylamide gel, and the gel was autoradiographed.

CDK2 Kinase Assay—NRK cells were suspended in hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 1 mM EDTA, 0.25 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM PMSF, 100 μg/ml leupeptin, 0.1% NP-40) for 20 min at 4°C. The samples were centrifuged at 10,000 × *g* for 10 min to remove the cytoplasmic fraction, and the pellet was resuspended in hypertonic buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 0.5 M NaCl, 25% glycerol, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 100 μg/ml leupeptin) for 20 min at 4°C. After centrifugation, the lysates were precleared by incubation with protein A-agarose beads, and the supernatant was incubated with anti-cyclin E for 2 h at 4°C. The immune complexes were collected on protein A-agarose beads and washed four times with the bead buffer (50 mM Tris-HCl [pH 7.4], 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 100 μg/ml leupeptin). The immunoprecipitates thus obtained were suspended in reaction buffer (20 mM HEPES [pH 7.4], 10 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 1 mM DTT) and incubated with histone H1 and [γ-^{32}P]ATP for 15 min at 30°C. The reaction was terminated by addition of the same volume of 2 × loading buffer, and the mixture was boiled for 5 min. The samples were electrophoresed on 12.5% polyacrylamide gel, and the gel was autoradiographed.

Western Blotting Analysis of Cyclins, CDK2, and pRB—NRK cells were harvested with PBS and sonicated in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween20, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4). The lysates were electrophoresed on 10% polyacrylamide gel (for CDK2, cyclin D and E) or 6.5% gel (for pRB) and then electrophoretically transferred to a PVDF membrane. The filter was blocked with 6% CS (for CDK2, and cyclins D and E) or 1% BSA (for pRB), washed, and incubated with a solution containing primary antibody. After having been washed, the filter was incubated with horseradish peroxidase-conjugated secondary antibody (Amersham). Finally, the protein bands were visualized with a Western Blot Chemiluminescence Reagent (Du Pont).

RESULTS

Effect of Inostamycin on MAP Kinase Activation—MAP kinase is known to be activated immediately by phosphorylation through the Ras cascade after serum addition (*36*). Activated MAP kinase can be seen as a band of lower electrophoretic mobility on Western blots (*37*). When quiescent NRK cells were stimulated with serum, MAP kinase was activated at 15 min. Although inostamycin at 1.25 μg/ml caused G_1 arrest, it did not inhibit serum-induced activation of MAP kinase at the same concentration, as shown in Fig. 1A. Additionally, inostamycin did not inhibit *in vitro* MAP kinase activity with myelin basic protein as a substrate (data not shown). Thus, inostamycin blocked serum-induced G_1 progression without affecting

the signaling pathway toward MAP kinase activation, most likely *via* Ras activation.

Effect of Inostamycin on CKII Activation—CKII is known to play a role in the transport of a mitogenic signal from the cytoplasm to the nucleus (*10*). The α-casein phosphorylation activity in NRK cells was enhanced from 15 min following serum addition, and this activity was completely inhibited by heparin, which is a specific CKII inhibitor (*38*) (Fig. 1B). This means that phosphorylation of α-casein in NRK cells could be due to CKII. Activation of CKII induced by serum was not affected by treatment with 1.25 μg/ml of inostamycin (Fig. 1B). Also, neither was serum-induced translocation of CKII to the nucleus inhibited by the same concentration of inostamycin (data not shown).

Inhibition of CDK2 Activation by Inostamycin—The CDK2/cyclin E complex has a histone H1 kinase activity that is thought to be necessary for cells to enter the S phase (*39*). The quiescent NRK cells began to enter the S phase in approximately 16 h with the peak at about 18 h after serum addition. Therefore, we examined the effect of inostamycin on CDK2 activation until 18 h after serum addition. As shown in Fig. 2A, histone H1 kinase activity in immune complexes prepared from NRK cells with anti-cyclin E was detected at 6 h after serum addition, and thereafter gradually increased as the cells reached the S phase. On the other hand, very weak histone H1 kinase activity of cyclin E immunoprecipitates prepared from the cells treated with 1.25 μg/ml of inostamycin was detected, even when the cells were stimulated with serum. Similar results were obtained when anti-CDK2 was used to form the immunoprecipitates (data not shown). Because inostamycin did not inhibit *in vitro* histone H1 phosphorylation catalyzed by CDK2/cyclin E complexes (data not shown), we examined the protein levels of CDK2 and cyclin E in NRK cells treated or not treated with 1.25 μg/ml of inostamycin. In quiescent cells, CDK2 proteins were detected as a single band, and were gradually phosphorylated on threonine 160, as detected by the shift of electrophoretic mobility, after serum addition. Inostamycin did not affect the total protein level of CDK2, but it did reduce the level of phosphorylated CDK2 (Fig. 2A). Expression of cyclin E, which was induced by serum at mid G_1 phase, was strongly inhibited by the treatment with inostamycin (Fig. 2A). Furthermore, the expression of cyclin E at 12 h after serum addition was inhibited dose-dependently by 0.125–1.25 μg/ml of inostamycin, and the inhibitory activity of inostamycin toward cyclin E expression corresponded with that toward CDK2 activity or S phase entry (Fig. 2, B and C).

Inhibition of Cyclin D1 Expression by Inostamycin—As inostamycin inhibited the synthesis of cyclin E, we examined the effect of inostamycin on the expression of an earlier G_1 cyclin, cyclin D1. As shown in Fig. 3A, cyclin D1 appeared at 3 h, prior to cyclin E expression, after serum stimulation. Inostamycin (1.25 μg/ml) also completely inhibited serum-induced cyclin D1 expression. Inhibition of cyclin D1 expression at 6 h after serum addition by inostamycin was dose-dependent over a similar dose range to

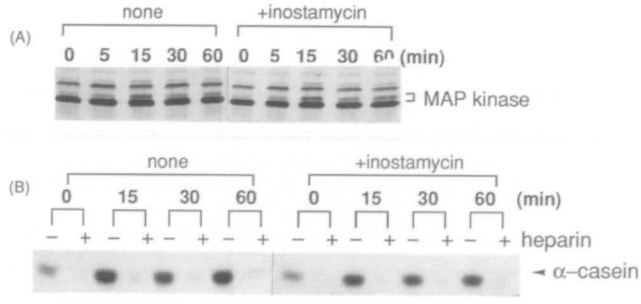

Fig. 1. **Effects of inostamycin on the serum-induced activation of MAP kinase and CKII in NRK cells.** Quiescent NRK cells were stimulated with 5% CS. Inostamycin at 1.25 μg/ml was added 15 min before the addition of the CS. The cells were collected at the indicated times and lysed. MAP kinase (A) and CKII (B) activities were assayed as described in "MATERIALS AND METHODS."

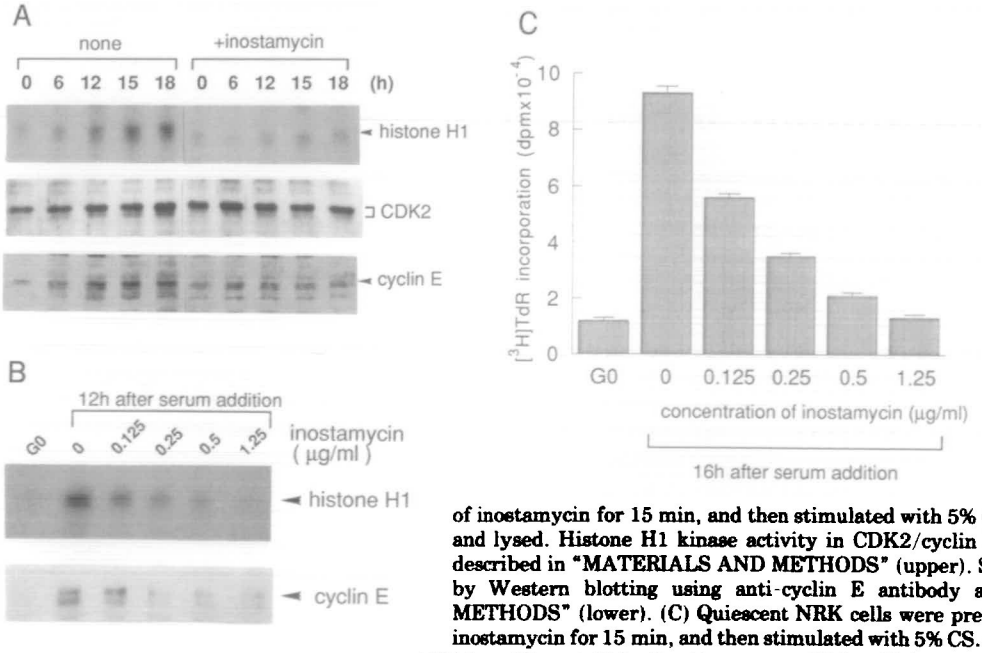

Fig. 2. **Inhibition of CDK2/cyclin E activation by inostamycin in NRK cells.** (A) Quiescent NRK cells were stimulated with 5% CS. Inostamycin at 1.25 μg/ml was added 15 min before the addition of the CS. Histone H1 kinase activity in CDK2/cyclin E immune complexes was assayed at the indicated times, as described in "MATERIALS AND METHODS" (upper). Samples (50 μg protein) were taken at the indicated times, and the expression levels of cyclin E (middle) and CDK2 (lower) were analyzed by Western blotting using their antibodies, as described in "MATERIALS AND METHODS." (B) Quiescent NRK cells were pretreated with various concentrations of inostamycin for 15 min, and then stimulated with 5% CS. After 12 h, the cells were collected and lysed. Histone H1 kinase activity in CDK2/cyclin E immune complexes was assayed as described in "MATERIALS AND METHODS" (upper). Samples (50 μg protein) were analyzed by Western blotting using anti-cyclin E antibody as described in "MATERIALS AND METHODS" (lower). (C) Quiescent NRK cells were pretreated with various concentrations of inostamycin for 15 min, and then stimulated with 5% CS. The cells were then labeled for 1 h with [^{3}H]thymidine at 16 h after the serum addition. Values are the means ± SD of triplicate samples.

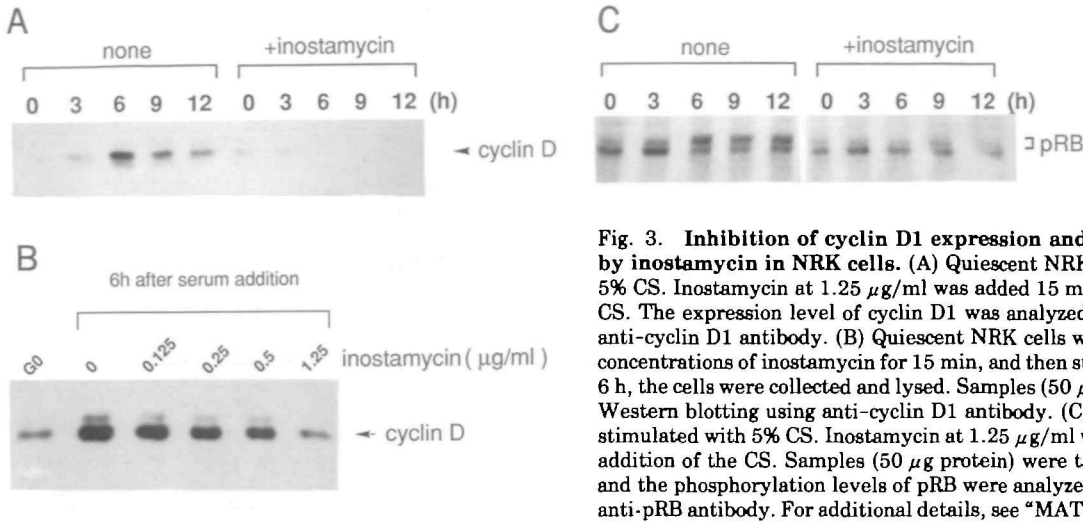

Fig. 3. **Inhibition of cyclin D1 expression and of pRB phosphorylation by inostamycin in NRK cells.** (A) Quiescent NRK cells were stimulated with 5% CS. Inostamycin at 1.25 μg/ml was added 15 min before the addition of the CS. The expression level of cyclin D1 was analyzed by Western blotting using anti-cyclin D1 antibody. (B) Quiescent NRK cells were pretreated with various concentrations of inostamycin for 15 min, and then stimulated with 5% CS. After 6 h, the cells were collected and lysed. Samples (50 μg protein) were analyzed by Western blotting using anti-cyclin D1 antibody. (C) Quiescent NRK cells were stimulated with 5% CS. Inostamycin at 1.25 μg/ml was added 15 min before the addition of the CS. Samples (50 μg protein) were taken at the indicated times, and the phosphorylation levels of pRB were analyzed by Western blotting using anti-pRB antibody. For additional details, see "MATERIALS AND METHODS."

that for inhibiting cyclin E expression (Fig. 3B). Cyclin D1 forms a complex with CDK4 (*20*), and this complex phosphorylates pRB, which is known to be a major substrate for the cyclin D1/CDK4 complex (*30, 40*). When serum was added to the serum-starved control cells, hyperphosphorylation of pRB, detected as a slower-migrating band in SDS-PAGE gels, increased gradually as cyclin D1 was expressed. As expected, inostamycin blocked this hyperphosphorylation of pRB (Fig. 3C).

DISCUSSION

A number of protein kinases are involved in signal transduction for cell proliferation mediated by serum and growth factors. Among them, MAP kinase and CKII are thought to be key regulatory proteins functioning in the signal transduction network. However, inostamycin did not affect the activation step of either enzyme. Since both enzymes are immediately activated in response to serum, these results are consistent with our previous finding that addition of inostamycin at 4 h after serum addition still caused G_1 arrest. On the other hand, inostamycin greatly lowered the histone H1 kinase activity of CDK2, although it did not affect the amount of CDK2. The activity of each CDK is regulated by different mechanisms. In the case of CDK2, its activation requires association with cyclin E (*24*). Moreover, complete activation also requires phosphorylation of the conserved threonine residue (Thr 160) by a CDK-activating kinase (CAK) (*41*). Inostamycin blocked induction of cyclin E expression, and consequently CDK2 could not form a complex with cyclin E, resulting in the inhibition of CDK2 activation. Phosphorylation of CDK2 was also inhibited by inostamycin. CAK phosphorylates monomeric CDK2 (*42*), and this phosphorylation may enhance the binding of some CDK/cyclin pairs (*43*). Conversely, cyclin binding may enhance phosphorylation (*25*). Since we did not exclude the possibility of CAK inhibition by inostamycin, it is still unclear whether inostamycin also inhibits CAK activation in addition to the inhibition of cyclin E expression for CDK2 phosphorylation.

CDK4 is activated by forming a complex with cyclin D1, and its activation is induced prior to that of CDK2. As inostamycin inhibits cyclin D1 expression, CDK4 activity is not activated. Indeed, serum-induced phosphorylation of pRB, which may be the rate-limiting substrate of the cyclin D1/CDK4 complex, was completely blocked by inostamycin. The data taken together demonstrate that inostamycin inhibits the expression of cyclins D1 and E, thereby causing cell cycle arrest at G_1 phase. These inhibitory effects should not be due to the toxic effect of the drug, because inostamycin-treated cells showed cyclin D1 expression and S phase entry at 6 and 18 h, respectively, after removal of the drug (data not shown). However, the mechanism of the inhibition by inostamycin of cyclin expression remains unclear. Moreover, it is also unclear whether inhibition of cyclin D1 expression by inostamycin results in a decrease in cyclin E expression or whether inostamycin inhibits signaling pathways leading to expression of both cyclins D and E.

Originally, inostamycin was isolated from microbial secondary metabolites as an inhibitor of CDP-DG:inositol transferase, which catalyzes phosphatidylinositol (PI) synthesis (*33*). The precise role of PI synthesis in signal transduction is still unknown. Previously, we reported that the dose range of inostamycin for inhibition of PI synthesis was closely related to that for inhibition of G_1 progression (*35*). Therefore, it is likely that PI synthesis is required for induction of G_1 cyclin expression. However, more direct inhibition of G_1 cyclin expression can not be ruled out.

Although various regulatory molecules for G_1 progression have been discovered recently, regulatory mechanisms for the expression of these molecules are not understood. Inostamycin provides a good tool to give us new insight into the regulatory mechanisms of G_1 progression.

REFERENCES

1. Pelech, S.L. and Sanghera, J.S. (1992) MAP kinases: Charting the regulatory pathways. *Science* **257**, 1355-1356
2. Cobb, M.H., Boulton, T.G., and Robbins, D.J. (1991) Extracellular signal-regulated kinases: ERKs in progress. *Cell Regul.* **2**, 965-978
3. Sturgill, T.W. and Wu, J. (1991) Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim. Biophys. Acta* **1092**, 350-357
4. Buday, L. and Downward, J. (1993) Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor,

Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* **73**, 611-620
5. Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* **363**, 85-88
6. Pawson, T. (1995) Protein modules and signalling networks. *Nature* **373**, 573-580
7. Crespo, P., Xu, N., Simonds, W.F., and Gutkind, J.S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature* **369**, 418-420
8. Koch, W.J., Hawes, B.E., Allen, L.F., and Lefkowitz, R.J. (1994) Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$. *Proc. Natl. Acad. Sci. USA* **91**, 12706-12710
9. Faure, M., Voyno-Yasenetskaya, T.A., and Bourne, H.R. (1994) cAMP and $\beta\gamma$ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* **269**, 7851-7854
10. Lorenz, P., Pepperkok, R., Ansorge, W., and Pyerin, W. (1993) Cell biological studies with monoclonal and polyclonal antibodies against human casein kinase II subunit β demonstrate participation of the kinase in mitogenic signaling. *J. Biol. Chem.* **268**, 2733-2739
11. Sommercorn, J., Mulligan, J.A., Lozeman, F.J., and Krebs, E.G. (1987) Activation of casein kinase II in response to insulin and to epidermal growth factor. *Proc. Natl. Acad. Sci. USA* **84**, 8834-8838
12. Klarlund, J.K. and Czech, M.P. (1988) Insulin-like growth factor I and insulin rapidly increase casein kinase II activity in BALB/c3T3 fibroblasts. *J. Biol. Chem.* **263**, 15872-15875
13. Ackerman, P. and Osheroff, N. (1989) Regulation of casein kinase II activity by epidermal growth factor in human A-431 carcinoma cells. *J. Biol. Chem.* **264**, 11958-11965
14. Carroll, D. and Marshak, D.R. (1989) Serum-stimulated cell growth causes oscillations in casein kinase II activity. *J. Biol. Chem.* **264**, 7345-7348
15. Yu, I.J., Spector, D.L., Bae, Y.-S., and Marshak, D.R. (1991) Immunocytochemical localization of casein kinase II during interphase and mitosis. *J. Cell Biol.* **114**, 1217-1232
16. Pepperkok, R., Lorenz, P., Ansorge, W., and Pyerin, W. (1994) Casein kinase II is required for transition of G_0/G_1, early G_1, and G_1/S phases of the cell cycle. *J. Biol. Chem.* **269**, 6986-6991
17. Russo, G.L., Vandenberg, M.T., Yu, I.J., Bae, Y.-S., Franza, B.R., Jr., and Marshak, D.R. (1992) Casein kinase II phosphorylates $p34^{cdc2}$ kinase in G_1 phase of the HeLa cell division cycle. *J. Biol. Chem.* **267**, 20317-20325
18. Pines, J. (1993) Cyclins and cyclin-dependent kinases: Take your partners. *Trends Biochem. Sci.* **18**, 195-197
19. Tassan, J.-P., Jaquenoud, M., Leopold, P., Schultz, S.J., and Nigg, E.A. (1995) Identification of human cyclin-dependent kinase 8, a putative protein kinase partner for cyclin C. *Proc. Natl. Acad. Sci. USA* **92**, 8871-8875
20. Xiong, Y., Zhang, H., and Beach, D. (1992) D-type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71**, 505-514
21. Meyerson, M. and Harlow, E. (1994) Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell Biol.* **14**, 2077-2086
22. Sherr, C.J. (1995) D-type cyclins. *TIBS* **20**, 187-190
23. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R., and Roberts, J.M. (1992) Formation and activation of cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. *Science* **257**, 1689-1694
24. Dulic, V., Lees, E., and Reeds, S.I. (1992) Association of human cyclin E with a periodic G_1-S phase protein kinase. *Science* **257**, 1958-1961
25. Morgan, D.O. (1995) Principles of CDK regulation. *Nature* **374**, 131-134
26. Mäkelä, T.P., Tassan, J.-P., Nigg, E.A., Frutiger, S., Hughes, G.J., and Weinberg, R.A. (1994) A cyclin associated with the CDK-activating kinase MO15. *Nature* **371**, 254-257
27. Fisher, R.P. and Morgan, D.O. (1994) A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* **78**, 713-724
28. Matsuoka, M., Kato, J., Fisher, R.P., Morgan, D.O., and Sherr, C.J. (1994) Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. *Mol. Cell. Biol.* **14**, 7265-7275
29. Devault, A., Martinez, A.-M., Fesquet, D., Labbe, J.-C., Morin, N., Tassan, J.-P., Nigg, E.A., Cavadore, J.-C., and Doree, M. (1995) MAT1 ('menage a trois'), a new RING finger protein subunit stabilizing cyclin H-CDK7 complexes in starfish and *Xenopus* CAK. *EMBO J.* **14**, 5027-5036
30. Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* **7**, 331-342
31. Sherr, C.J. (1994) The ins and outs of RB: Coupling gene expression to the cell cycle clock. *Trends Cell Biol.* **4**, 15-18
32. Nevins, J.R. (1992) E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**, 424-429
33. Imoto, M., Taniguchi, Y., and Umezawa, K. (1992) Inhibition of CDP-DG:inositol transferase by inostamycin. *J. Biochem.* **112**, 299-302
34. Imoto, M., Taniguchi, Y., Fujiwara, H., and Umezawa, K. (1994) Enhancement of CDP-DG:inositol transferase activity in *src*- and *erbB2*-transformed cells. *Exp. Cell Res.* **212**, 151-154
35. Imoto, M., Morii, T., Deguchi, A., and Umezawa, K. (1994) Involvement of phosphatidylinositol synthesis in the regulation of S phase induction. *Exp. Cell Res.* **215**, 228-233
36. Crews, C.M. and Erikson, R.L. (1993) Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell* **74**, 215-217
37. Leevers, S.J. and Marshall, C.J. (1992) Activation of extracellular signal-regulated kinase, ERK2, by $p21^{ras}$ oncoprotein. *EMBO J.* **11**, 569-574
38. Hathaway, G.M., Lubben, T.H., and Traugh, J.A. (1980) Inhibition of casein kinase II by heparin. *J. Biol. Chem.* **255**, 8038-8041
39. Sherr, C.J. (1993) Mammalian G1 cyclins. *Cell* **73**, 1059-1065
40. Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J., and Kato, J. (1994) D-Type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell Biol.* **14**, 2066-2076
41. Fesquet, D., Labbe, J.-C., Derancourt, J., Capony, J.-P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M., and Cavadore, J.-C. (1993) The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J.* **12**, 3111-3121
42. Poon, R.Y.C., Yamashita, K., Adamczewski, J.P., Hunt, T., and Shuttleworth, J. (1993) The cdc2-related protein $p40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $p33^{cdk2}$ and $p34^{cdc2}$. *EMBO J.* **12**, 3123-3132
43. Desai, D., Wessling, H.C., Fisher, R.P., and Morgan, D.O. (1995) Effects of phosphorylation by CAK on cyclin binding by CDC2 and CDK2. *Mol. Cell Biol.* **15**, 345-350