Inhibition of the Association with Nuclear Matrix of pRB, p70 and p40 Proteins Along with the Specific Suppression of c-MYC Expression by Geldanamycin, an Inhibitor of Src Tyrosine Kinase

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Geldanamycin is an antibiotic that preferentially inhibits G1/S transition and causes G2/M arrest in human leukemia HL-60 cells. With it, we selectively inhibited recombinant Src tyrosine kinase without significantly inhibiting protein kinase A. The perturbation of cell cycling by geldanamycin was accompanied by marked suppression of c-MYC expression.

In contrast to this, pRB expression was remarkably enhanced by geldanamycin. In the untreated HL-60 cells, c-MYC was apparently enriched in nuclear matrix preparation, and significant amounts of hyperphosphorylated pRB, p70 and p40 proteins were observed to associate with the nuclear matrix. The amounts of these proteins associated with the nuclear matrix, however, were markedly decreased by treatment with geldanamycin. This finding suggests that the association of c-MYC, hyperphosphorylated pRB, p70 and p40 proteins with the nuclear matrix is essential in cell cycling, especially in G1/S and G2/M progressions, and that this association is a part of signal transduction pathway in Src kinase activation.

Several lines of evidence indicate that progression through the cell cycle is regulated by the signal transduction induced by ligand interactions with cell surface receptors[1]. Receptor-linked or membrane associated protein tyrosine kinase has been designated as one of the most important frames in the signal transduction for cell proliferation. Recent studies have begun to unravel the intracellular signaling molecules involved in cell cycling[2]. The commitment of the cell to enter S phase is ensured by the initiation of DNA replication. The involvement of oncogene or tumor suppressor gene expression in DNA replication has been argued concerning differentiation and malignant transformation of mammalian cells. Knowledge of the mechanisms that initiate DNA replication in eukaryotic cells is, however, very limited.

A variety of experiments indicate that c-MYC expression is essential for progression of S phase[3]. The molecular basis of the involvement of c-MYC in DNA replication is still not fully understood; although ARIGA *et al.* have described c-MYC's participation in the process[4]. Our previous evidence that geldanamycin specifically inhibited c-MYC expression along with the inhibition of DNA replication could support their findings[5]. v-MYC expression, however, was observed to antagonize differentiation[6]. It has been also reported that c-MYC is required for terminal differentiation of cells[7,8]. Furthermore, EVAN and coworkers have described apoptosis by c-MYC overexpression[9]. Human c-MYC is a protein of 67 kDa with common features of the transcription factor: leucine zipper (LZ) domain, helix loop helix (HLH) domain, and phosphorylation sites by casein kinase II (CKII). It has binding sites for the tumor suppressor *Rb* protein (pRB) and MYC associated protein X (MAX). The pRB interferes with the transcription by c-MYC[10], and MAX activates the transcription by forming the MYC/MAX heterodimer[11].

The expression of c-MYC is an immediate early response, like FOS and JUN expressions[12]. The c-MYC expression is activated by such growth factors as serum, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) or 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) through the protein kinase C (PKC) pathway. It is also activated by mitogenic stimuli, such as concanavanin A, lipopolysaccharides, and phytohemaggulutinin. Thus, c-MYC expression is presumed to

be regulated by a variety of signalling pathways.

Here, we present the possibility that the inactivation of the Src family tyrosine kinase associated with plasma membrane interferes with the signal transduction, which confers early responsive c-MYC expression by means of a specific inhibitor of Src tyrosine kinase, geldanamycin (GDM) (Fig. 1)[13,14].

GDM is an antibiotic of the benzoquinoid ansamycin group, and was isolated as a product of *Streptomyces* sp. in a screening program to isolate anticancer substances.

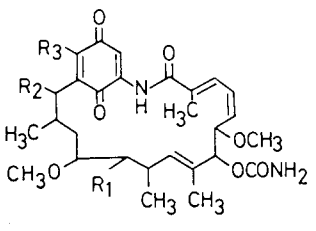
The antibiotic showed preferential inhibition of the transition from G1 to S phase of the cell cycle (G1/S transition) and caused G2/M arrest in the HL-60 human leukemia cells along with specific suppression of c-MYC expression accompanied by overexpression of pRB. We also found that four proteins—c-MYC, hyperphosphorylated pRB (ppRB), p70 and p40— were associated with the nuclear matrix, and an association was clearly inhibited by GDM. The results suggest that the association of these protein with the nuclear matrix actively contributes to G1/S and G2/M progression. We present here the importance of activating Src family tyrosine kinase for c-MYC and pRB expression regulation and their association with the nuclear matrix in cell cycling.

Materials and Methods

Materials

Geldanamycin (GDM) was a generous gift from Nisshin Flour Milling Co. Ltd., Saitama-ken, Japan. Herbimycin A (HBA) was kindly provided by Dr. S. ŌMURA, Institute of Kitasato, Tokyo. Anti-mouse Ig rabbit globulin conjugated with horse radish peroxydase (HRP) and Western blot Enhanced chemiluminisence (ELC) kit were products of Amersham International plc, Buckinghamshire, England. [γ-^{32}P]ATP (111 TBq/mmol) was from Dupont-New England Nuclear Co. Ltd., Wilmington, DE.; anti-human c-MYC (Clone 9E10)[15] and anti-pRB (Clone C36)[16] monoclonal antibodies were purchased from Oncogene Science Inc., Uniondale, NY., IgG-Sepharose 6FF was from Pharmacia Co. Ltd., Piscataway, NJ.; poly(Glu - Tyr (4 : 1)), cAMP-dependent protein kinase (protein kinase A) and propidium iodide were from Sigma Chemical Co., St. Louis, MO. RNase UK-9 was from Miles Lab.,Co. Ltd. UK. EPICS 750 (Coulter Co., Hialeah, FL) was used for flow cytometry analysis.

Fig. 1. The chemical structures of geldanamycin and herbimycin A.



	R1	R2	R3
Geldanamycin	-OH	-H	-OCH_3
Herbimycin A	-OCH_3	-OCH_3	-H

Cells

HL-60 human leukemia cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum in a humidified CO_2 incubator (5% CO_2, 95% air) at 37°C.

Flow Cytometry Analysis

Ten milliliters of HL-60 cells were cultured at an initial density of 1.5×10^5 cells/ml of 10 ml in a 100-mm culture dish for 20 hours with or without GDM. The cells were harvested, washed with PBS three times, fixed with 1 ml of 70% ethanol, and washed three times with TN buffer (0.1 M Tris-HCl (pH 7.2), 0.1 M NaCl). The cells were then treated with 1 ml of RNase (1 mg/ml of UK-9 in PBS), washed three times with TN buffer, and stained with propidium iodide.

Isolation of Nuclei, Nuclear Extracts and Matrices

Whole cells were suspended in PMSF buffer I (50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 M sucrose and 1% Nonidet P-40 (NP-40)) and Dounce-homogenized 10 strokes. Nuclei were pelleted by centrifugation at 2000 rpm for 10 minutes. Pellets were placed in a Eppendorf tube and mixed 100 μl of PMSF buffer II (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM PMSF and 1% NP-40). The nuclei suspension was sonicated for 1 minute, and centrifuged at 15,000 rpm for 20 minutes. The resulting supernatants and precipitates were designated as nuclear extracts and matrices, respectively. The nuclear matrices were washed once with PMSF buffer II.

Immunoblotting

A 15-μl aliquot of nuclear extracts or matrices was mixed with an equal volume of a twofold higher concentration of SDS sample buffer (4% SDS, 0.001% bromophenol blue, 100 mM Tris-HCl (pH 6.8), 20% glycerol, 500 mM 2-mercaptoethanol (2-ME)) and boiled for 5 minutes and then applied to SDS gel electrophoresis. Proteins including c-MYC or pRB were detected by western immunoblot procedure. The proteins blotted on a sheet of nitrocellulose by electrotransfer were first reacted with anti-human c-MYC (clone 9E10) or anti-pRB (clone C36) mAb, then reacted with anti-mouse Ig rabbit globulin conjugated with HRP, and finally reacted with ECL reagents and exposed on a Kodak X-ray film.

Reaction of Protein Kinases

Reaction of Src tyrosine kinase. The truncated Src protein fused to protein A coded in the expression vector[17] was provided from transfected *E. coli*. Bacterial cells transfected with the expression vector were grown in Luria-Bertani (LB) medium containing 50 μg/ml of ampicillin, harvested by centrifugation, suspended in ice-cold RIPA A buffer (20 mM sodium phosphate (pH 7.4), 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate), and sonicated on ice using a UDC-200T Bioruptor/Cellruptor. After centrifugation at 10,000 $\times g$ for 10 minutes at 4°C, the supernatant was harvested and incubated with IgG-Sepharose 6FF at 4°C for overnight. After three washes with buffer (10 mM sodium phosphate (pH 7.4), 150 mM NaCl and 0.1% Triton X-100), the pellet was suspended in tyrosine kinase buffer (50 mM HEPES (pH 7.5), 0.1 mM EDTA and 0.015% Brij 35) containing 0.1 mg/ml bovine serum albumin (BSA) and 0.2% 2-ME was used as an enzyme source. The reaction mixture contained 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.015% Brij 35, 50 μM ATP, 10 mM $MgCl_2$, 0.07% 2-ME, 33 μg/ml BSA, 40 μg poly(Glu/Tyr (4 : 1)) and 370 kBq [γ-^{32}P]ATP in a total volume of 100 μl, and it was incubated at 30°C for 2 hours. The supernatant from the reaction mixture was collected and mixed with 100 μl of 5 mg/ml BSA and 500 μl of TCA solution (20% TCA (w/v), 20 mM NaH_2PO_4). After standing on ice for 30 minutes, the TCA-insoluble fraction was sedimented for 15 minutes at 10,000 $\times g$ and washed five times with the TCA solution. The radioactivity of the pellet dissolved in 0.2 M Tris-HCl (pH 8.0) was quantitated by liquid scintillation counting.

Reaction of protein kinase A. The reaction of protein kinase A was performed essentially according to method described previously[18]. A reaction mixture in a total volume of 70 μl contained 12 mM of potassium phosphate (pH 6.8), histone IIS 500 μg, 5 mM Mg acetate, 0.25 mM [γ-^{32}P]ATP (specific activity 350 pBq/mmole), 6 μM cAMP and 150 units of cAMP-dependent protein kinase from rabbit muscle (Sigma Co. Ltd.), and was incubated for 20 minutes at 30°C. A 50-μl aliquot of the reaction mixture was blotted onto a phosphocellulose paper disk, washed with H_2O five times, and the radioactivity was quantitated by liquid scintillation counting.

Results

Effect of GDM on Protein Kinases

It is assumed that Src tyrosine kinase is a major target site of GDM because cells transformed with v-*src* are selectively sensitive to the antibiotic, and the autophosphorylation of Src tyrosine residues in intact cells is specifically inhibited by it[14]. Here, we demonstrated that GDM specifically inhibits Src tyrosine kinase without inhibiting protein kinase A. We used a recombinant Src kinase fused to protein A, purified by complexing with IgG-Sepharose[17]. The Src kinase conjugated to IgG-Sepharose was used for assay reaction. GDM inhibited the recombinant Src kinase to a larger extent than herbimycin A, a specific inhibitor of Src kinase[14,20] (Table 1). The IC_{50} of GDM and herbimycin A were 0.35 μg/ml and 7.0 μg/ml, respectively. Protein kinase A from rabbit muscle was used to examine the effect of GDM on the phosphorylation of ser/thr protein residues. No significant inhibition of protein kinase A by GDM was observed (Table 2).

Effect of GDM on Cell Cycle Progression

The analysis by flow cytometry of cell-cycle progression in HL-60 cells with or without GDM is presented in Fig. 2. The G0/G1 phase population of cells accumulated and those of S phase completely depleted in the presence of GDM indicates that the antibiotic preferentially blocks the cell from entering S phase.

Alternative G2/M phase arrest was observed in the presence of GDM, indicating that termination of DNA replication or segregation of chromosomes is also blocked by GDM.

Inhibition of c-MYC Expression, Enhancement of pRB Expression, and Inhibition of their Association with the Nuclear Matrix by GDM

The above experiments indicate that GDM blocks the

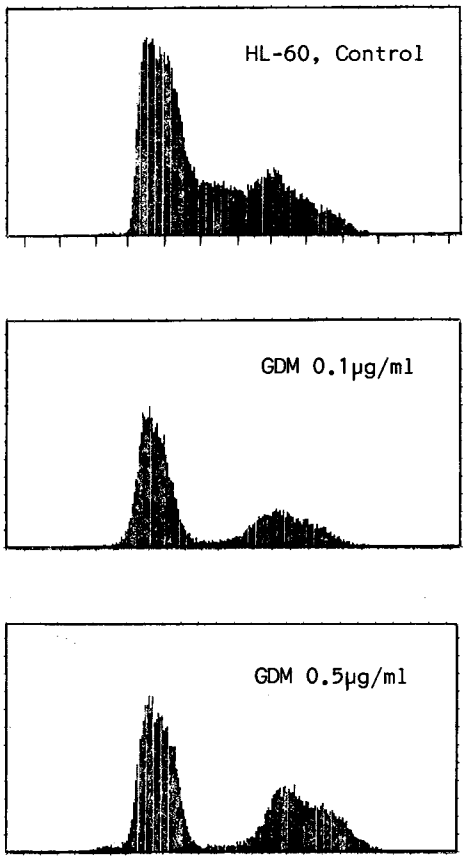
Table 1. Inhibition of Src tyrosine kinase by geldanamycin and herbimycin A.

drug (μg/ml)	dpm	(% incorporation)
None	3838	100
GDM 10	522	13.6
GDM 1	932	24.3
GDM 0.1	3106	81.0
HBA 50	516	13.4
HBA 5	2125	55.4
HBA 0.5	2888	75.3

Table 2. The effect of GDM on protein kinase A.

GDM (μg/ml)	dpm	(% incorporation)
None	6443	100
56	5346	83
5.6	5606	87
0.56	6260	97

Fig. 2. Effect of GDM on the cell-cycle progression of HL-60 cells.

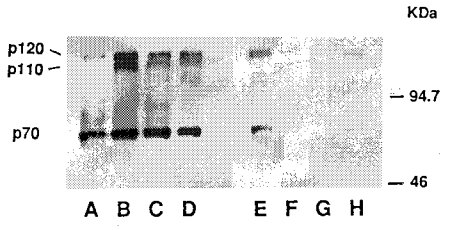


Flow cytometry analysis of HL-60 cells cultured at initial density of 1.5×10^5 cells/ml by treatment with GDM 0.1 μg/ml or 0.5 μg/ml for 20 hours are compared with that of the untreated control cells.

Fig. 3. Effects of GDM on c-MYC and p40 expression and their association with the nuclear matrix in HL-60 cells.

The proteins transferred from a polyacrylamide gel to a nitrocellulose sheet were analyzed by western-blot analysis. The c-MYC and 40 kDa protein cross-reactive with anti-human c-MYC mAb are indicated by p67 and p40, respectively. Lanes A to D indicate nuclear extracts, and lanes E to H inicate nuclear matrices from HL-60 cells untreated (control) (A, E) and those treated with GDM 1 μg/ml (B, F), 0.2 μg/ml (C, G) and 0.04 μg/ml (D, H). Molecular weights of marker proteins are indicated in kilodaltons.

Fig. 4. Effects of GDM on pRB and p70 expressions and their association with the nuclear matrix in HL-60 cells.



The proteins transferred from a polyacrylamide gel to a nitrocellulose sheet were analyzed by western-blot analysis. The pRB and 70 kDa protein cross-reactive with anti-pRB mAb are indicated by p110 (underphosphorylated pRB), p120 (hyperphosphorylated pRB) and p70. Lanes A to D indicate nuclear extracts, and lanes E to F indicate nuclear matrices from HL-60 cells untreated (control) (A, E), and those treated with GDM 1 μg/ml (B, F), 0.2 μg/ml (C, G) and 0.04 μg/ml. Molecular weights of marker proteins are indicated in kilodalton.

expression of a certain initiator of DNA replication. Previous studies suggest that c-MYC localized in nuclei is a factor in G1/S transition of mammalian cells[3]. The association of c-MYC with the nuclear matrix has been reported to be enhanced in the S phase of the cell cycle[19]. Thus, we examined the effect of GDM on the expression of c-MYC localized in nuclei and its association with the nuclear matrix in HL-60 cells. Nuclear extracts and matrices were prepared from HL-60 cells in the presence for 4 hours or absence of GDM. c-MYC was detected by immunoblot analysis with an anti-human c-MYC mAb (clone 9E10). This mAb recognized two major proteins of 67 kDa and 40 kDa on immunoblot analysis of the nuclear extracts and matrices of HL-60 cells. GDM was found to specifically inhibit the expression of c-MYC and consequently to decrease its localization on the nuclear matrix (Fig. 3). The antibiotic did not inhibit the expression of another 40-kDa protein (p40) which was cross-reactive with the anti-c-MYC mAb but decreased the amount of p40 associated with the nuclear matrix. The p40 is yet uncharacterized except for certain homology to the LZ domain of c-MYC[15]. The anti-pRB mAb (clone C36) recognized three major proteins of 110 kDa (pBB) and 120 kDa (ppRB), and another 70-kDa protein (p70) on immunoblot analysis. A homology search revealed that the p70 was possibly identical to lamin A[20] of which is known to localize in nuclei. The appearance of pRB in the nucleus was markedly enhanced by treatment with GDM, as shown in Fig. 4. The expression of p70 was also increased slightly in the presence of the antibiotic, and ppRB and p70 were observed to associate with the nuclear matrix. c-MYC, ppRB and p70 are likely to associate tightly with nuclear

matrix. It was of particular interest that ppRB was found to be associated with the nuclear matrix (underphosphorylated pRB was not associated with it). The association of these proteins with the nuclear matrix was preferentially inhibited by GDM accompanied by the suppression of c-MYC expression and the overexpression of pRB.

Discussion

The inhibition by GDM of Src family kinase is likely responsible for prohibiting cells to transform or to proliferate by means of the cascade pathway of signal transduction to the nucleus mediated by signal transducers and activators of transcription pathway. We demonstrated here the marked suppression of c-MYC expression accompanied by enhancement of pRB expression in the presence of GDM. It is conceivable that c-MYC and pRB are coordinatively expressed and function through the cell cycle, indicating that the inhibition by GDM of Src family tyrosine kinase expressed in HL-60 cells signalled the specific suppression of the early responsive c-MYC expression along with the enhanced pRB expression, thus committing the cells to G0/G1 and G2/M arrest.

We are also aware of the possibility that the expression or functional activation of intracellular signalling molecules involved in cell cycling, such as cyclin-dependent kinase (CDK) and cyclins, is affected by GDM. It has been shown that cyclin A and E expressions are under the control of MYC expression[22]. CDK2-cyclin E complex and CDK2, CDK4 or CDK5 complexed with cyclin D2 catalyze pRB phoshorylation, releasing it and enabling it to promote G1 exit[23]. It is possible that GDM inhibits c-MYC expression followed by the downregulation of cyclin A and E expressions, and thereby the phosphorylation of pRB can be prevented. Actually, the amounts of underphosphorylated pRB preferentially increased in the presence of GDM (Fig. 4), suggesting that phosphorylation of pRB is prevented by GDM, possibly indirectly. We can speculate then that the accumulation of underphosphorylated pRB in nucleus by GDM inhibits cells entering S phase. The molecular basis of this inhibition might be clarified by analyzing the function of pRB/c-MYC complex. Our results show that the four proteins—c-MYC, ppRB, p70 and p40—were associated with the nuclear matrix but that the association disappeared when GDM was added. This finding indicates that the nuclear matrix provides a place for the association of a multiprotein complex of factors involved in the regulation of cell cycling.

The molecular events of chromosomal DNA replication and chromosomal segregation in mammalian cells remain obscure. It has been suggested that the attachment of c-MYC to the nuclear matrix is possibly involved in S phase progression[19], or nuclear structural organization[24]. The possible functions of replisome attachment to membranous scaffold[25] may be provided in our studies by the plausible notion that the supermolecular assembly of replisome exists in association with the initiation, termination in DNA replication or segregation of chromosome in mammalian cells. Our results show that GDM inhibits G1/S transition and G2 or M traverses of cells in cell cycle progression and interferes with the attachment to the nuclear matrix of c-MYC and ppRB, which are thought to be regulators of DNA replication. The results suggest that the association of c-MYC with the nuclear matrix is concerned with its functioning as an initiator of DNA replication or in segregation of chromosomes.

The importance of attachment of the bacterial chromosomal replication origin (oriC) region to membrane structure in the regulation of DNA replication and cell division has been persuasively documented[26~31].

We present here evidence that ppRB (but not underphosphorylated pRB) is tightly associated with the nuclear matrix, and this association was markedly blocked by GDM along with G0/G1 and G2/M arrests. Therefore, we speculate that the association of ppRB, c-MYC, p70 or p40 with the nuclear matrix is essential to override the inhibition of either G1 exit to initiate DNA replication or G2/M exit to segregate chromosomes.

A search for the mechanism of action of the antitumor antibiotic, GDM, an inhibitor of Src tyrosine kinase, will give us a clue to understanding the molecular mechanisms of the signal transduction pathway to cell cycling in malignant transformed cells. Thus, GDM could be a valuable tool to clarify the molecular events that could reveal how the association of certain proteins with the nuclear matrix contributes to the cell cycle progression of mammalian cells.

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