S6K1 Is Involved in Polyploidization Through Its Phosphorylation at Thr421/Ser424

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Studies on polyploidization of megakaryocytes have been hampered by the lack of synchronized polyploid megakaryocytes. In this study, a relatively synchronized polyploid cell model was successfully established by employing Dami cells treated with nocodazole. In nocodazole-induced cells, cyclin B expression oscillated normally as in diploid cells and polyploid megakaryocytes. By using the nocodazole-induced Dami cell model, we found that 4E-BP1 and Thr421/Ser424 of ribosomal S6 kinase 1 (S6K1) were phosphorylated most at M-phase in cytoplasm and oscillated in nocodazole-induced polyploid Dami cells, concomitant with increased expression of p27 and cyclin D3. However, phosphorylation of 4E-BP1 and S6K1 on Thr421/Ser424 was significantly decreased in differentiated Dami cells induced by phorbol 12-myristate 13-acetate (PMA), concomitant with increased expression of cyclin D1 and p21 and cyclin D3. Overexpression of the kinase dead form of S6K1 containing the mutation Lys 100 → Gin in PMA-induced Dami cells increased ploidy whereas overexpression of rapamycin-resistant form of S6K1 containing the mutations Thr421 → Glu and Ser424 → Asp significantly dephosphorylated 4E-BP1 and reduced expression of cyclin D1, cyclin D3, p21 and p27, and slightly decreased the ploidy of PMA-induced Dami cells, compared with treatment with PMA alone. Moreover, overexpression of rapamycin-resistant form of S6K1 significantly reversed polyploidization of nocodazole-induced Dami cells. Furthermore, MAP (a novel compound synthesized recently) partly blocked the phosphorylation of S6K1 on Thr421/Ser424 and decreased the expression of p27 and polyploidization in nocodazole-induced Dami cells. Taken together, these data suggested that S6K1/4E-BP1 pathway may play an important role in polyploidization of megakaryocytes.


Terminal differentiation of megakaryocytes is characterized by nuclear polyploidization, cell size growth, and generation of additional cell surface and cytoplasmic protein that are required for platelet formation and function (Hoffman, 1989; Avraham, 1993). Polyploidization is a phenomenon unique to megakaryocytes among hematopoietic cells, but observable in almost all organisms. It can occur during reproduction, differentiation, or tumor progression (Nagl, 1990). Although it has been known that endomitosis, a process of successive rounds of DNA synthesis in the absence of cytokinesis, is a major cause of polyploidization in these cells, the underlying mechanism is largely unknown (Brodsky and Uryvaeva, 1977). It is believed that endomitosis benefits the process of platelet production because it enables megakaryocytes to increase their transcriptional activity and supports the massive increase in cellular mass (Hancock et al., 1993). This is critical for platelet heomostasis as large highly polyploid megakaryocytes produce highly reactive and larger platelets than their smaller, low ploidy counterparts (Zimmet and Ravid, 2000). Therefore, an attempt to understand the mechanism of this process is of both clinical and scientific significance.

It has been proposed that endomitosis in megakaryocyte is attribute to DNA synthesis without mitosis, that is, that megakaryocytes undergo several rounds of replication separated by gaps. However, some investigations have suggested that endomitosis represents an aborted mitosis (Naga, et al., 1997; Vitrat et al., 1998). Nevertheless, most investigations about the regulation of endomitosis have focused on the regulation of cell cycle transitions during G2/M.

Some investigations on yeast and megakaryocytic leukemia cell lines have shown that endomitosis may be due to a lack of (or reduction in) cyclin B1 or cdc2, leading to a decrease in mitotic kinase activity (Broek et al., 1991; Datta et al., 1996; Zhang et al., 1996, 1998; Matsumura et al., 2000). However, studies on human and murine megakaryocyte progenitor cells have demonstrated that abundant cyclin B1 was expressed in 8N-32N megakaryocytes during G2/M (Vitrat et al., 1998; Carow et al., 2001). The level of cyclin B1 per G2/M megakaryocyte did not reduce, but increased linearly with ploidy and the expression oscillated normally in polyploid megakaryocytes (Carow et al., 2001).

The D-type cyclins (D1, D2, and D3) with lineage-dependent expression, in conjunction with their catalytic partners cdk4 and cdk6, control cell cycle progression from G1 to S phase. To prevent abnormal proliferation, cdk–CDK complexes are precisely regulated by two families of cell cycle inhibitors that block their catalytic activity. The first class of inhibitors includes the INK4 proteins that bind only to cdk4–cdk6 kinases and not to cyclins and are therefore specific for early G1 phase. The second family of inhibitors is composed of Cip/Kip proteins, such as p21, p27 and p57 that inhibit all cdk–CDK complexes and are not specific for a particular phase. Unlike INK4 proteins, Cip/Kip proteins do not dissociate cyclin–CDK complexes (Sherr and Roberts, 1995). The distribution of cyclin–CDK complexes between cytoplasm and nucleus is also regulated by the intracellular level of inhibitors of the Cip/Kip family (Coqueret, 2003).
Both cyclin D1 and cyclin D3 are involved in promoting polyploidization of megakaryocyte. Cyclin D3 and, to a much lesser extent, cyclin D1, are present in megakaryocytes undergoing endomitosis and these cyclins are, respectively, markedly and moderately up-regulated following exposure to the ploidy-promoting factor, TPO (Zimmet et al., 1997; Ma et al., 2000). Moreover, overexpression of cyclin D1 and cyclin D3 results in an increased ploidy (Zimmet et al., 1997; Sun et al., 2001). In addition, cyclin D3 is only located in cytoplasm of TPO-induced fetal megakaryocytes, which are with low ploidy (>4N) and in both cytoplasm and nuclei of TPO-induced adult megakaryocytes (Ma et al., 2000). Furthermore, the nuclear localization of cyclin D3 was increased with PD98059 treatment and inhibited by rapamycin (Guerriero et al., 2006). The molecular mechanisms and signaling pathways inducing megakaryocytic cyclin D3 nuclear translocation still remains largely unknown.

It has been shown that high levels of p21Cip1/Waf1 and p27Kip1 are expressed on megakaryocytes during differentiation (Zimmet et al., 1998; Taniguchi et al., 1999a). Overexpression of p21 resulted in an increase in ploidy of megakaryocytic cell lines (Kikuchi et al., 1997; Matsumura et al., 1997) and TPO induced p21 transcription and expression through signal transducer and activator of transcription 5 (Stat5) activation (Matsumura et al., 1997). These data suggested that p21 is implicated in megakaryocyte polyploidization. However, recently, it has been shown that overexpression of p21 in p21−/− or normal murine megakaryocytes and in human megakaryocytes results in a marked inhibition in megakaryocyte polyploidization in all these cases (Baccini et al., 2001) and decrease in mean ploidy level and the delay in megakaryocyte differentiation induced by rapamycin were less pronounced in Cdkn1a (p21)−/− megakaryocytes than in Cdkn1a (p21)+/+ megakaryocyte (Raslova et al., 2006), indicating that other members of the Cip/Kip family may play a role in megakaryocyte polyploidization.

Studies on human polyploidization of megakaryocytes have been hampered by the lack of pure and abundant megakaryocyte progeny and synchronized polyploidy megakaryocytes. Although polyploidization is concomitant with maturation, they are two distinct processes during differentiation (Kikuchi et al., 1997). Therefore, a relative synchronized polyploid cell model with less differentiation and a more differentiated cell model with less polyploidization may be helpful to exploring the molecular mechanisms and signaling pathways. In this investigation, we optimized these two models in Dami cells (a megakaryocytic leukemia cell line) induced by nocodazole and phorbol 12-myristate 13-acetate (PMA) respectively. By using these models, the role of the mammalian target of rapamycin (mTOR), the ribosomal S6 kinase 1 (S6K1), the eukaryotic initiation factor 4E (eIF4E), 4E binding protein 1 (4E-BPI), was investigated during ployploidization.

Materials and Methods

Reagents

Nocodazole was purchased from Sigma (St. Louis, MO), diluted at 50 μg/ml in dimethylsulfoxide (DMSO) and stored at −20 °C until being used. Phorbol 12-myristate 13-acetate (PMA) from Sigma, was diluted to 1 mg/ml in DMSO and aliquots of 0.05 ml were frozen at −80 °C. Rapamycin (FRAP/mTOR inhibitor), LY294002 (PI3 kinase inhibitor), wortmannin (all from Calbiochem, San Diego, CA) and MAP, a new organic chemical synthesized in our laboratory (for application for patent, the data about MAP is not published) were dissolved in DMSO and aliquots were frozen at −80 °C. S6K1 antibody, phospho-S6K1 (Thr389) antibody, phospho-S6K1 (Thr421/Thr422) antibody, S6 ribosomal protein antibody, phospho-S6 ribosomal protein (Ser240/244) antibody, phospho-S6 ribosomal protein (Ser235/236) antibody, elf4E antibody, phospho-elf4E (Ser209) antibody, phospho-4E-BPI (Thr37/46) antibody, phospho-4E-BPI (Ser65) antibody, phospho-4E-BPI (Thr70) antibody, cdck2 antibody, phospho-cdc2 (Tyr15) antibody, p44/42 MAP kinase antibody, phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, β-actin antibody, goat-rabbit IgG conjugated with HRP, and LumiGLOTM reagent were purchased from Cell Signaling Technology. 4E-BP1 (R-113) rabbit polyclonal antibodies, cyclin B1 rabbit polyclonal antibodies, cyclin D1 rabbit polyclonal antibodies, cyclin D3 rabbit polyclonal antibodies, p21 rabbit polyclonal antibodies and p27 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell lines and culture conditions

Dami cells were grown in Iscove’s modified Dulbecco’s medium (IMDM, Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS) (Gibco BRL). Cultures were maintained in a humidified atmosphere of 5% CO2 at 37 °C. To determine growth curve, cells were seeded at 5 × 10^3/ml in 10 ml of complete media and cultured in 25 cm² tissue culture flask (Costar, Corning Incorporated, Corning, NY). Cells counts were made using the trypsin blue dye exclusion method.

Treatment of cells

Dami cells seeded at 5 × 10^3/ml were treated with PMA at different concentration from 5 to 20 ng/ml or with nocodazole at different concentration from 12.5 to 200 ng/ml for specified times respectively, and DMSO was adjusted to 0.25% of the final concentration. To remove the adherent cells from the flasks, cultures were washed once with 3 ml PBS and treated with 3 ml PBS plus 2.5 mM EDTA. Cultures were incubated for 5 min at 37 °C and washed three times with PBS. Viable cells accessed by the trypsin blue dye exclusion method. In some experiments, 2 h prior to the addition of nocodazole, rapamycin (50 nM), LY294002 (10 μM), wortmannin (1 μM), and MAP (10 ng/ml) were added to the culture. Cells were fixed with 80% methanol for 10 min on ice to shear DNA and reduce sample viscosity. Cell lysates were cleared by centrifugation at 10,000 rpm for 5 min at 4 °C. The protein concentration was measured by means of BCA protein assay reagent kit (PIERCE Biotechnology, Rockford, IL) to ensure equal electrophoretic treatment and inhibited by rapamycin. Luminograms were prepared by exposing immunoblots to humidiX-ray film.

Morphological analysis

Cells collected at different days of culture were cytocentrifuged onto glass slides, stained with Wright-Giemsa and then identified by morphological analysis.

Western blot analysis

Cells were collected and washed with PBS at 1,400 rpm for 5 min at 4 °C. The cells pellets were lysed with sample buffer (Tris–HCl: 62.5 mM, Glycerol: 10%, SDS (w/v): 10%) containing 1 mM PMSF and 50 mM DTT and sonicated for 6 min on ice to shear DNA and reduce sample viscosity. Cell lysates were cleared by centrifugation at 10,000 rpm for 5 min at 4 °C. The protein concentration was measured by means of BCA protein assay reagent kit (PIERCE Biotechnology, Rockford, IL) to ensure equal electrophoretic loading. Lysates were loaded on 6–12% SDS–PAGE gel and transferred to a nitrocellulose filter. Blots were blocked using 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 2 h at room temperature. The membranes were incubated overnight at 4 °C with different primary antibodies. After three washes in TBS, the membranes were incubated with goat-rabbit IgG conjugated to HRP at room temperature for 2 h. Reactive proteins were detected by LumiGLOTM reagent and luminograms were prepared by exposing immunoblots to X-ray film.
Ploidy analysis and immunofluorescence

Cells fixed with 80% methanol were maintained for at least 24 h at −20 °C and washed in PBS containing 1% BSA and then permeabilized with 0.25% Triton X-100 for 4 °C for 15 min. After being washed at 1,300 g, cells were incubated at 4 °C overnight with anti-cyclin B1 rabbit polyclonal antibody (1:200) or anti-phospho-S6K1 (Thr421/Ser424) rabbit polyclonal antibody (1:100) or anti-phospho-4E-BP1 (Thr37/46) rabbit polyclonal antibody (1:100) or anti-phospho-4E-BP1 (Ser65) rabbit polyclonal antibody (1:100), respectively. Rabbit IgG was used as a control. The cells were then washed twice and incubated with FITC-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA) for 30 min at 4 °C. Some cells were labeled with phospho-histone H3 (Ser10) antibody conjugated with Alexa Fluor 488 (1:10). Rabbit IgG conjugated with Alexa Fluor 488 was used as negative control. DNA was stained with PBS containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A (Sigma) for 2 h. Cells were analyzed on FACSort flow cytometer (Becton Dickinson).

For each sample, 10,000 cells were acquired. The data were analyzed using the Cellquest software package.

Immunolabeling for microscopy and digital images were prepared with Adobe photoshop

Cells were centrifuged onto glass slides at 800 rpm for 5 min with a Labofuge 400R centrifuge (Heraeus Instruments, Heraus, Germany). The cells were fixed with 3% paraformaldehyde in PBS for 30 min at 4 °C. After being washed three times for 5 min each with PBS, cells were incubated with PBS containing 0.3% Triton X-100 and 2% normal goat serum. After being washed, the cells were incubated with anti-phospho-S6K1 (Thr421/Ser424) rabbit polyclonal antibody (diluted 1:50), or anti-phospho-4E-BP1 (Thr37/46) rabbit polyclonal antibody (1:50) or anti-phospho-4E-BP1 (Ser65) rabbit polyclonal antibody (1:50). The cells were then washed twice and incubated with FITC-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA) for 30 min at 4 °C. Some cells were labeled with phospho-histone H3 (Ser10) antibody conjugated with Alexa Fluor 488 (1:10). Nuclei were visualized using To-Pro3 (1:1,000, Molecular Probes Inc., Eugene, OR). The slides were mounted in ProLong Antifade (Molecular Probes Inc.) and imaged with a Leica TCS SP II laser scanning confocal microscope. Digital images were prepared with Adobe photoshop.

Transfection of Dami cells with plasmids and analysis with flow cytometry and Western blot

Dami cells were transiently transfected with pRKS-myc-S6K1 (wild-type S6K1, WT), myc-d/ED3E-pRKS (rapamycin-resistant mutant, RR) containing the mutationsSer11 → Asp, Ser148 → Asp, Thr421 → Glu and Ser424 → Asp and Thr389 → Glu, or myc-2BQ-pRKS (kinase Dead, KD) containing Lys 100 → Gin recombinant plasmids. These plasmids were kindly provided by Dr. Thomas and had been described elsewhere (Ming et al., 1994; Pearson et al., 1995). Briefly, Dami cells were washed once in cold phosphate-buffered saline and resuspended in the specified electroporation buffer to a final concentration of 1.2 × 10^6 cells/ml. Two micrograms of each of plasmids (described above) was mixed with 0.1 ml of cell suspension. The mixture was transferred to a 2.0 mm electroporation cuvette, and nucleofected with an Amaxa Nucleofector™ apparatus (Amaxa, Cologne, Germany). After transfection, the cells were resuspended in IMDM medium with 10% FCS and PMA (10 ng/ml) and incubated in a humidified atmosphere of 5% CO2 at 37 °C. After 3-day incubation, some of the transfected cells were lysed with sample buffer and analyzed with Western blot as described above. The other part of the transfected cells were fixed with 1% paraformaldehyde at 4 °C for 1 h. After being washed with PBS, the cells were permeated with 70% ethanol and stained with PBS containing 50 μg/ml PI and 100 μg/ml RNase A for 2 h. For each sample, 10,000 cells were acquired by using FACSort. The data were analyzed using the Cellquest software package. In some experiments, the Dami cells were transfected with myc-d/ED3E-pRKS and treated with nocodazole (50 ng/ml) and then incubated in a humidified atmosphere of 5% CO2 at 37 °C. After 3-day incubation, the transfected cells were processed for analysis of FACSort and Western blot as described above.

Results

The effect of Nocodazole and PMA in inducing polyploidization of Dami cells

A previous investigation showed that nocodazole and 12-O-tetradecanoylphorbol-13-acetate (TPA) induced polyploidization and differentiation respectively (Kikuchi et al., 1997). Therefore, in this study, nocodazole and PMA were employed to induce Dami cells. Nocodazole inhibited the growth of Dami cells at 50 ng/ml with 87% of Dami cells excluding trypan blue after 3-day induction (data not shown). The percentage of polyploid cells (≥8N) increased from 5.3% ± 1.2% at day 0 to 69.7% ± 10.2% at day 3 in the presence of nocodazole (Fig. 1A) (Table 1). Morphologically, M-phase arrest was seen at day 1, and mature megakaryocyte-like polyploid cells with less cytoplasm were detected after day 2 (Fig. 1B). Therefore, nocodazole was used at 50 ng/ml in following experiments. PMA inhibited the growth of Dami cells at 5, 10, or 20 ng/ml with >95% of Dami cells excluding trypan blue after 3-day induction (data not shown). The different concentrations of PMA had a similar effect on the ploidy distribution of Dami cells as well, but the effect of PMA was much weaker than that of nocodazole (Fig. 1C). PMA at 10 ng/ml slightly increased the proportion of polyploid cells from 5.3 ± 1.2% at day 0 to 10.7 ± 1.4% at day 3 (Table 1).

Morphologic analysis showed that the PMA-induced polyploid cells were with more cytoplasm (Fig. 1D). Therefore, in the following experiments, PMA was used at 10 ng/ml. Analysis of phenotype showed that PMA up-regulates megakaryocytic marker, CD61, and down-regulates the erythrocytic marker, glycophorin A. Although nocodazole had a similar effect on differentiation of Dami cells towards megakaryocytic lineage, it was much weaker than that of PMA (Fig. 1E). Since thrombopoietin (TPO) induced megakaryocytic differentiation through up-regulation of cyclin D1 (Matsumura et al., 2000), the expressions of cyclins, CDKs, and CKIs involved in megakaryocyte polyploidization were investigated. As shown in Figure 1F, nocodazole increased the expressions of cyclin B1, cyclin D3 and p27 whereas PMA upregulated cyclin D1, cyclin D3 and p21. Although both nocodazole and PMA increased the expression of cdc2, the up-regulation of cdc2 induced by nocodazole was limited between 2N and 4N cell cycle (see Fig. 3B for cell cycle analysis). In addition, PMA induced dephosphorylation of cdc2 on tyrosine 15. However, phosphorylation of cdc2 on tyrosine 15 remained until 60 h after nocodazole induction, indicating that cdc2 was inhibited during polyploidization induced by nocodazole. The data indicated that nocodazole and PMA induced a relatively synchronized polyploidization and differentiation in Dami cells through regulation of different protein expressions and phosphorylation, respectively.

Cyclin B was expressed in a cell cycle-specific manner in Dami cells induced by both nocodazole and PMA

In normal diploid cells, cyclin B expression began at S-phase, reached maximal levels at G2/M, and declined during anaphase.
when cyclin B is degraded by the cyclin-specific proteasome (Pines and Hunter, 1989; Darzynkiewicz et al., 1996). It was also found that the expression of cyclin B was low in G1 phase, increased in S phase populations and reached maximal levels at G2/M in murine megakaryocytes induced by TPO, suggesting cell cycle-specific oscillation in megakaryocytes (Carow et al., 2001). In order to determine if the expression of cyclin B in polyploid Dami cells induced by nocodazole or PMA oscillated within polyploid cell cycle as in megakaryocytes, the cells were stained with anti-cyclin B1 and propidium iodide (PI). Although the effect of PMA on induction of polyploidization of Dami cells was much weaker than that of nocodazole, both PMA and nocodazole induced cyclin B1 expression in Dami cells as cell cycle-specific oscillation (Fig. 2), which was the same as the expression of cyclin B1 in polyploid megakaryocytes (Carow et al., 2001). Cyclin B1 expression increased in S phase populations and reached maximal levels at G2/M. When the cells progressed into the polyploid cell cycle, cyclin B1

Fig. 1. Effect of nocodazole and PMA on Dami cells. Dami cells seeded at $5 \times 10^5$/ml in IMDM containing 10% FCS were treated with nocodazole and PMA respectively for different periods. The distribution of ploidy was analyzed by flow cytometry (A,C). Morphologic analysis was estimated based on Wright-Giemsa staining of each cytocentrifuged preparation (original magnification 1,000 x) (B,D). Dami cells treated with nocodazole or PMA and harvested at day 3 were stained with anti-Glycophorin A (GLY A)-FITC (dark line in the left column of the histogram part) or anti-CD61-FITC (dark line in the right column of the histogram part) and analyzed by flow cytometry (E). FITC-labeled normal isotype mouse IgG was used as negative control (light line in histograms). Lysates obtained from Dami cells induced with nocodazole or PMA for the times indicated in hour (h) were run on SDS-PAGEs and the membranes were probed with specific antibodies against cyclin B1, cyclin D1, cyclin D3, p-cdc2, cdc2, p21, p27 or β-actin (F). A representative experiment was shown. Similar results were obtained in other three independent experiments.
expression decreased in G1 phase, increased in S phase and reached maximal levels at G2/M. Notably, the majority of nocodazole-treated Dami cells had relatively the same DNA contents after 1-day or 2-day or 3-day induction (see the left column in Fig. 2A). However, polyploidization-inducing effect of PMA was much weaker than that of nocodazole, that is, almost half of the cells had 2N DNA content and the other half had 4N DNA content after 1-day or 2-day or 3-day induction of PMA (see the left column in Fig. 2B). These data suggested that nocodazole-induced Dami cells would relatively mimic the endomitosis of megakaryocytes in a more synchronized way.

**S6K1 and 4E-BP1 were phosphorylated by nocodazole but not by PMA**

Since nocodazole and PMA regulated different protein expressions, expression and phosphorylation of mTOR pathway proteins, which were involved in translation, were investigated with Western blots, and parallel experiments monitored the cell cycle stage of polyploidization by flow cytometry. Both nocodazole and PMA had no significant effect on the expression and phosphorylation of akt although PMA slightly increased the phosphorylation of akt at Ser473. In nocodazole-induced Dami cells, the expression of mTOR and the phosphorylation of mTOR at Ser2448 increased every 12 h (12, 36, and 60 h) (Fig. 3A). Analysis with flow cytometer showed that Dami cells begin to progress from G1 to S phase in diploid cell cycle and polyploid cell cycle at these time points (Fig. 3B). However, PMA had no significant effect on the expression and phosphorylation of mTOR. Nocodazole significantly increased the long term phosphorylation of 4E-BP1 at Thr37/46 and Ser65, and S6K1 at Thr421/Ser424 in both diploid cell cycle and polyploid endocycle although the phosphorylation of S6K1 at Thr389 was not affected by the treatment. PMA significantly reduced phosphorylation of S6K1 at Thr 389 and had no obvious effect on the phosphorylation of 4E-BP1 at Ser65. As shown in Figure 5B, most cells labeled with phospho-histone H3 (Ser10) antibody, which detects cells in M-phase, was employed to label nocodazole-induced Dami cells with different antibodies against phosphorylated S6K1 at Thr421/Ser424 or phosphorylated 4E-BP1 at Thr37/46 and Ser65 were phosphorylated. Since the overlap of DNA content between G2/M cells of any given polyploid class and G1 cells of the next ploidy class (Gong et al., 1993), we further investigated in which of the phase of the polyploid cell cycle S6K1 at Thr421/Ser424, and 4E-BP1 at Thr37/46 and Ser65 were phosphorylated. It was found that phosphorylation of histone H3 at Ser 10 was mainly at M-phase except a few at late G2 in mammalian cells with site-specific antibody highly specific for the phosphorylated form of the amino-terminus of histone H3 (Ser10) (Hendzel et al., 1997). In this study, we further demonstrated that the phosphorylation of histone H3 (Ser 10) was also observed at M-phase of polyploid Dami cells (Fig. 5A). Therefore, Alexa Fluor® 488 conjugated phospho-histone H3 (Ser10) antibody, which detects cells in M-phase, was employed to label nocodazole-induced Dami cells with different antibodies against phosphorylated S6K1 at Thr421/Ser424 or phosphorylated 4E-BP1 at Thr37/46 or phosphorylated 4E-BP1 at Ser65. As shown in Figure 5B, most cells labeled with phospho-histone H3 (Ser10) antibody were also labeled with anti-phospho-p70 S6 (Thr421/Ser424) antibodies (91.54%) or anti-phospho-4E-BP1 (Thr37/46) antibody (77.36%) or anti-phospho-4E-BP1 (Ser65) antibody (80.28%). Each cell cycle was divided into several phases on the basis of DNA content, and these phases were identified on the basis of DNA content (%): G0/G1 cells with less than diploid DNA content, S phase cells with DNA content between diploid and tetraploid DNA content, G2/M phase cells with more than tetraploid DNA content (Fig. 4A). As the Dami cells began to enter polyploidization under the induction of nocodazole, these phosphorylation oscillated from G2/M-phase at 4N to G2/M-phase at 8N and then to G2/M at 16N gradually. However, PMA significantly reduced the phosphorylation of S6K1 and 4E-BP1 at these specific sites in polyploid Dami cells at G2/M-phases, especially after 3-day treatment with PMA, although PMA slightly induced Dami cells into polyploidization (Fig. 4B).

**S6K1 and 4E-BP1 were phosphorylated in a cell cycle-specific manner**

Since mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1. DNA and phosphorylated S6K1 or phosphorylated 4E-BP1 double-labeling techniques were employed to determine in which phase of cell cycle of polyploidization these proteins were phosphorylated. Analysis of flow cytometry showed that phosphorylation of S6K1 at Thr421/Ser424, 4E-BP1 at both Thr37/46 and Ser65 were specifically labeled by their associated antibodies at G2/M-phase in untreated diploid Dami cells (Fig. 4A). As the Dami cells began to enter polyploidization under the induction of nocodazole, these phosphorylation oscillated from G2/M-phase at 4N to G2/M-phase at 8N and then to G2/M at 16N gradually. However, PMA significantly reduced the phosphorylation of S6K1 and 4E-BP1 at these specific sites in polyploid Dami cells at G2/M-phases, especially after 3-day treatment with PMA, although PMA slightly induced Dami cells into polyploidization (Fig. 4B).

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**TABLE 1. Ploidy distribution of Dami cells induced by Nocodazole and PMA (%)**

<table>
<thead>
<tr>
<th>Time of treatment</th>
<th>2N (%)</th>
<th>4N (%)</th>
<th>8N (%)</th>
<th>16N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>56.9 ± 2.7</td>
<td>36.8 ± 3.8</td>
<td>5.3 ± 1.0</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Day 1</td>
<td>9.2 ± 6.3</td>
<td>80.5 ± 10.1</td>
<td>8.7 ± 2.9</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>Day 2</td>
<td>9.1 ± 7.5</td>
<td>38.3 ± 6.2</td>
<td>47.7 ± 12.8</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Day 3</td>
<td>8.5 ± 7.6</td>
<td>22.7 ± 7.0</td>
<td>56.2 ± 11.6</td>
<td>12.5 ± 4.8</td>
</tr>
</tbody>
</table>

The ploidy was accessed with flow cytometry following DNA staining with propidium iodide (PI). Ten thousand cells were analyzed on each sample. The data were obtained from four independent experiments and shown as mean ± SEM values.
Fig. 2. Expression of cyclin B1 in nocodazole-induced and PMA-induced Dami cells. The Dami cells treated with nocodazole (A) or PMA (B), were harvested at day 0, 1, 2, and 3. The cells were stained with anti-cyclin B1 antibody or isotype-matched control. DNA was labeled with PI. Cells were analyzed using flow cytometry and the data were evaluated with CELL Quest software.
Thr421/Ser424 may be involved in polyploidization through translation.

**Mutant S6K1 plasmids increased ploidy of PMA-induced Dami cells**

In order to prove the hypothesis, PMA-induced Dami cells were transfected with pRK5-myc-S6K1 (wild-type S6K1, WT), myc-d/ED3E-pRK5 (rapamycin-resistant mutant, RR) and myc-2BQ-pRK5 (kinase dead, KD) as described in Materials and Methods Section. PMA-induced Dami cells were used as control. The ploidy of transfected cells were analyzed with flow cytometer. As shown in Figure 6A, overexpression of pRK5-myc-S6K1 (WT) and myc-2BQ-pRK5 (KD) increased the ploidy of PMA-induced Dami cells significantly, compared with PMA-induced Dami cells. The proportion of the cells with DNA content ≥8N in PMA-induced population was 11.62%. In contrast, the proportion of the cells with DNA content ≥8N in pRK5-myc-S6K1 (WT) and myc-2BQ-pRK5 (KD)-transfected populations treated with PMA were 32.36% and 43.5%, respectively, indicating that the effect of myc-2BQ-pRK5 (KD) on induction of ploidy was much stronger than that of pRK5-myc-S6K1 (WT). However, overexpression of myc-d/ED3E-pRK5 (RR) did not change the ploidy of PMA-induced Dami cells significantly, the proportion of the cells with DNA content ≥8N in myc-d/ED3E-pRK5 (RR)-transfected populations was 10.65%.

Since S6K1 mediated assembly of the translation initiation complex through the eIF3-PIC (translation preinitiation complex) served as a dynamic scaffold (Holz et al., 2005), the activation of S6K1 downstream target were analyzed. Western blot analysis showed that although ectopic expression of S6K1 among the transfected cells with pRK5-myc-S6K1 (WT), myc-2BQ-pRK5 (KD) or myc-d/ED3E-pRK5 (RR) were...
different as detected with myc-tag antibody and S6K1 antibody (Fig. 6B), expression of the phospho-S6K1 at Thr421/Ser424 and phospho-S6K1 at Thr 389 in the cells transfected with pRK5-myc-S6K1 (WT), myc-2BQ-pRK5 (KD) or myc-d/ED3E-pRK5 (RR) increased significantly, compared with that in PMA-induced Dami cells. The expression of the phospho-S6K1 at Thr421/Ser424 and phospho-S6K1 at Thr 389 in the transfected cells with pRK5-myc-S6K1 (WT) or
Fig. 5. S6K1 and 4E-BP1 of polyploid Dami cells was phosphorylated simultaneously with phosphorylation of histone H3 on Ser 10 at M-phase in cytoplasm. (A) Dami cells were treated with nocodazole and harvested at the indicated days. Double-labeling with anti-phospho-histone H3 (Ser10) antibody conjugated with Alexa Fluor® 488 and PI were performed as described in Materials and Methods Section. Rabbit IgG conjugated with Alexa Fluor® 488 was used as negative control. 10,000 cells were analyzed. (B) The nocodazole-induced Dami cells harvested at day 3 were labeled with anti-phospho-histone H3 (Ser10) antibody and anti-phospho-S6K1 (Thr421/Ser424) antibody or anti-phospho-4E-BP1 (Thr37/46) antibody or anti-phospho-4E-BP1 (Ser65) antibody simultaneously as described in Materials and Methods Section. (C) Dami cells were treated with nocodazole and harvested at day 3. Cells on slides were stained with anti-phospho-S6K1 (Thr421/Ser424) antibody (a) or anti-phospho-4E-BP1 (Thr37/46) antibody (e), combined with TRITC-conjugated secondary antibody, and Alexa Fluor® 488 conjugated Phosphor-Histone H3 (Ser10) antibody (b,f). Nuclei were visualized using To-Pro3 (c,g). TRITC, Alexa Fluor® 488, and To-Pro3 fluorescence were photographed separately and the images were overlaid to form a composite (d,h), showing triple stainings of the same cells.
myc-2BQ-pRK5 (KD) were much higher that in the transfected cells with myc-d/ED3E-pRK5 (RR) although the expression of myc-d/ED3E-pRK5 (RR) was highest among the three Dami cell population transfected with pRK5-myc-S6K1 (WT), myc-2BQ-pRK5 (KD) or myc-d/ED3E-pRK5 (RR) as detected with myc-tag antibody and S6K1 antibody. These results are ascribe to that myc-d/ED3E-pRK5 (rapamycin-resistant mutant, RR) contains the mutations Ser411 → Asp, Ser418 → Asp, Thr421 → Glu and Ser424 → Asp and Thr389 → Glu and that phospho-S6K1 (Thr389) antibody and phospho-S6K1 (Thr421/Ser424) antibody could not recognized the mutant S6K1 protein efficiently. However, expression of s6

Fig. 6. Effect of mutant S6K1 plasmids on ploidy of PMA-induced Dami cells and Nocodazole-treated Dami cells. Dami cells were transiently transfected with various myc-tagged S6K1 plasmids respectively and refed with IMDM containing 10% FBS in the presence of PMA or ncodazo as described in Materials and Methods Section. After 3 days incubation, the cells were processed for flow cytometer analysis and Western blot analysis. The DNA histograms were obtained from Dami cells (control), PMA-induced Dami cells (PMA), and PMA-induced Dami cells transfected with pRK5-myc-S6K1 (PMA + WT), myc-d/ED3E-pRK5 (PMA + RR) or myc-2BQ-pRK5 (PMA + KD) differently (A). Lysates obtained were run on SDS–PAGEs and the blots were probed with specific antibodies against myc-tag, p-S6K1 (Thr389), p-S6K1 (Thr421/Ser424), S6K1, p-S6 (Ser240/244), p-S6 (Ser235/236), S6, p-4E-BP1 (Thr37/46), p-4E-BP1 (Ser65), p-4E-BP1 (Ser70), 4E-BP1, cyclin D1, cyclin D3, p21 and p27 (B, C). DNA histograms obtained from Dami cells (control), nocodazole-induced Dami cells (NO), and nocodazole-induced Dami cells transfected with myc-d/ED3E-pRK5 (NO + RR) were stained with PI and analyzed by a flow cytometer (D).
ribosomal protein and phosphorylation of s6 ribosomal protein on Ser235/236 and Ser240/244 did not change among the three infected cell populations. In contrast, overexpression of myc-d/ED3E-pRKS (RR) significantly reduced expression of 4E-BP1 and phosphorylation of 4E-BP1 at Thr34/46 and Ser65 and Ser70 although overexpression of pRKS-myc-S6K1 (WT) slightly decreased expression of 4E-BP1 and dephosphorylated 4E-BP1 at Thr34/46 and Ser65 and Ser70, compared with untreated Dami cells (Fig. 6D). However, overexpression of myc-2BQ-pRKS (KD) did not decrease the expression of 4E-BP1 and phosphorylation of 4E-BP1 at Thr34/46 and Ser65 and Ser70 in PMA-induced cells. Furthermore, overexpression of myc-d/ED3E-pRKS (RR) also significantly reduced expression of cyclin D1, cyclin D3, p21 and p27, especially cyclin D1 and p21, although overexpression of pRKS-myc-S6K1 (WT) slightly decreased cyclin D1, cyclin D3 and p21 as well (Fig. 6C). These data suggested that phosphorylation of S6K1 on Thr421/Ser424 may regulate polyploidization positively through translation.

In order to further confirm the hypothesis, nocodazole-treated Dami cells were transfected with myc-d/ED3E-pRKS (RR). As presumed, overexpression of myc-d/ED3E-pRKS (RR) significantly reduced the ploidy of nocodazole-treated Dami cells (Fig. 6D). Overexpression of myc-d/ED3E-pRKS (RR) reduced the proportion of high ploidy (>8N) Dami cells induced with nocodazole from 56.45% (nocodazole-treated alone) to 32.56% (nocodazole-treated in combination with transfection with myc-d/ED3E-pRKS (RR) plasmids). Overexpression of myc-d/ED3E-pRKS (RR) significantly dephosphorylated 4E-BP1 at Thr34/46, Ser65 and Ser70 and reduced expression of cyclin D1, cyclin D3, p21 and p27 in nocodazole-induced Dami cells as well (data not shown).

MAP partly blocked the phosphorylation of S6K1 at Thr421/Ser424 and decreased polyploidization in nocodazole-induced Dami cells

In order to block the phosphorylation of S6K1 at Thr421/Ser424, 2 h prior to the addition of nocodazole, rapamycin (50 nM), LY294002 (10 μM), wortmannin (1 μM), and MAP (1 ng/ml) were added to the culture. Surprisingly, MAP partly blocked polyploidization and inhibited the phosphorylation of S6K1 at Thr421/Ser424 and decreased the expression of p27 in Dami cells induced by nocodazole without affecting the phosphorylation of S6 (Fig. 7). However, rapamycin, LY294002, and wortmannin had no significant effect on the phosphorylation of S6K1 at Thr421/Ser424 and the expression of p27 in Dami cells induced by nocodazole (data not shown).

Discussion

In the past, the study of megakaryocytic polyploidization was hampered by the rarity of megakaryocytes in normal bone marrow (only 0.03–0.06% of the nucleated cells), poorly defined cell populations, and inadequate assay methods (Hoffman 1989; Avraham, 1993). A previous investigation revealed that treatment with the microtubule depolymerizing agent, nocodazole, preferentially induced polyploidization of UT-7 cells with a relatively small increase in differentiation and TPA provoked a dramatic increase in differentiation with much less effect on polyploidization (Kikuchi et al., 1997). However, UT-7 cells were fragile when treated with nocodazole. In this study, a relatively synchronized polyploid cell model with less differentiation and a more differentiated cell model with less polyploidization were successfully established by employing Dami cells treated with nocodazole or PMA.

The mTOR pathway is involved in the control of mammalian cell size (Fingar et al., 2002) and the regulation of cell growth and proliferation by means of activating the G1/S transition (Fingar et al., 2004). It was previously demonstrated that TPO-induced PI3K activity in megakaryocyte progenitors (Geddiss et al., 2001) and that a decrease in TPO-dependent AKT and S6K1 was induced by rapamycin (Rapa), mTOR inhibitor, in the hematopoietic UT7-mpl cell line expressing exogenous TPO receptors (Nagata and Todokoro, 1995). Recent investigations showed that TPO induced the phosphorylation of mTOR and its effector proteins, S6K1 and 4E-BP1, and the inhibition of the mTOR pathway by Rapa resulted in a reduction in both cell proliferation and polyploidization (Guerriero et al., 2006; Raslova et al., 2006). However, no significant difference was detected in the mean ploidy level between a control culture and the treated culture when the addition of Rapa was delayed, indicating that the effect of Rapa on polyploidization may be indirect, and through the inhibition of the G1/S transition in proliferative progenitors (Raslova et al., 2006). Megakaryocyte precursors switch toward an endomitotic process characterized by a series of discrete G1-S-G2 phases. Each G2 phase is followed by an incomplete mitosis leading to the absence of karyokinesis and cytokinesis. To our knowledge, it is not clear whether the mTOR pathway is involved in G2/M phase regulation during polyploidization until now.

In this study, we found that the expression and phosphorylation of mTOR not only increased during progression from G1 to S phase in diploid cell cycle, but also in the transition from G1 to S phase of polyploid endocycle, indicating that mTOR played an important role in progression from G1 to S phase in both diploid cell cycle and polyploid cell cycle. It was noteworthy that the phosphorylation of S6K1 at Thr421/Ser424 and the phosphorylation of 4E-BP1 at Thr34/46 and Ser65 and Ser70 were significantly increased at M phase in relatively synchronized polyploid Dami cells induced by nocodazole, which oscillated with the increment of ploidy as well as the expression of cyclin B1. In contrast, the phosphorylation of S6K1 and 4E-BP1 was significantly reduced at G2/M phases in more differentiated Dami cells induced by PMA although some of PMA-induced Dami cells had polyploid DNA contents (>4N). Moreover, overexpression of the kinase dead form of S6K1 (myc-2BQ-pRKS, KD) in PMA-induced Dami cells increased polyploidy whereas overexpression of the rapamycin-resistant form of S6K1 did not. Given that rapamycin-resistant form of S6K1 (myc-d/ED3E-pRKS, RR) contains the mutations Thr421→Glu, Ser424→Asp and Thr389→Glu which confers rapamycin resistance on the kinase and negates the inhibitory effects of the macrolide on S6K-mediated control of translation was thought to occur through phosphorylation of the 40S ribosomal protein S6. The increase in S6 phosphorylation was proposed to govern the translation of a specific subset of mRNAs containing 5'-terminal oligopyrimidine (5'-TOP) tracts encoding ribosomal components and translation elongation factors (Jefferies et al., 1997). In this investigation, surprisingly, we found that the S6 phosphorylation of polyploid Dami cells induced with nocodazole significantly decreased whereas S6 phosphorylation of Dami cells induced with PMA increased. Moreover, Ectopic overexpression of kinase dead form of S6K1 (myc-2BQ-pRKS, KD) in PMA-induced Dami cells did not change S6 phosphorylation whereas the ploidy of PMA-induced Dami cells was increased. Thus, these results indicated that phosphorylation of S6 was not linked to polyploidization.
Fig. 7. MAP partly blocked the phosphorylation of S6K1 at Thr421/Ser424 and decreased the expression of p27 with reduction of polyploidization. After treatment with MAP for 2 h, Dami cells seeded at 2.5 × 10^5/ml in IMDM containing 10% FCS were induced with nocodazole at 50 ng/ml and harvested at the indicated days. Lysates obtained were run on three separate SDS-PAGEs. The blots were probed with specific antibodies against S6K1, p-S6K1 (Thr421/Ser424), S6, S6 (Ser235/236), and p27 (A). The cells harvested at the indicated day were also stained with PI and analyzed with flow cytometry (B).
control, which is consistent with the finding by Pende et al. (2004) that phosphorylation of S6K1, but not S6, was linked to cellular growth control.

A recent investigation showed that the eif3-PIC (translation preinitiation complex) served as a dynamic scaffold for mTOR- and S6K1-mediated assembly of the translation initiation complex (Holz et al., 2005). Interaction between S6K1 and the eif3-PIC was governed by the activating phosphorylation of S6K1 on the hydrophobic-motif residue Thr389. The phosphorylation of Thr389 was critical for the release of S6K1 from the eif3 complex. Although it was not known whether the phosphorylation of Thr421/Ser424 leads to release of S6K1 from the eif3 complex or not, the phosphorylation of these sites led to a conformational change of S6K1 (Pullen and Thomas, 1997), which may disrupt S6K1 and eif3-PIC binding. Furthermore, nocardazole induced the expression of p27 and cyclin D3, which promoted polyploidization of megakaryocytes. Overexpression of rapamycin-resistant form of S6K1 (myc-d/ED3E-pRKS, RR) significantly dephosphorylated 4E-BP1 at Thr37/46 and Ser40, reduced expression of cyclin D1, cyclin D3, p21 and p27, and reduced the ploidy of nocardazole-treated Dami cells. Therefore, we presumed that nocardazole increased the expression of the p27 and cyclin D3 through activation of S6K1/4E-BP1 pathway, which mediated assembly of the translation initiation complex, to promote polyploidization. This presumption was further confirmed also by partly blocking the phosphorylation of S6K1 at Thr421/Ser424 with MAP, which was concomitant with decrease of both the polyploidy and the expression of p27.

It is noticeable that overexpression of pRKS-myc-65K1 (WT) increased the ploidy although expression and phosphorylation of 4E-BP1 were decreased and the protein levels of cyclin D1, cyclin D3, p21 and p27 was reduced slightly in PMA-induced cells transfected with pRKS-myc-S6K1 (WT), compared with Dami cells treated with PMA alone. This result suggested that other mechanism or molecules might be involved to regulate ployploidization.

Nocardazole is a microtubule-disrupting agent which affects the microtubule and thus impairs cytokinesis without affecting karyokinesis. During polyploidization, primary megakaryocytes enter mitosis and progress through normal prophase, prometaphase, metaphase, and up to anaphase A, but not to anaphase B, telophase, or cytokinesis (Nagata et al., 1997; Vitrat et al., 1998) while in both nocardazole- and PMA-induced polyploidization, the cells are multinucleated (the karyokinesis is not affected) (Fig. 1B, D). In addition, Dami cells is a leukemic cell line in which the JAK2/STAT5 signal transduction pathway was constitutively activated, likely ascribing to JAK2 V617F tyrosine kinase mutation and leading to factor-independent growth (Liu et al., 1999; James et al., 2005; Quentmeier et al., 2006). With these in mind, therefore, the model used in this work (induction of ploidy and differentiation by nocardazole and PMA, respectively) is not completely identical to the physiological polyploidization of primary megakaryocytes.

However, Nocardazole and PMA not only just blocked the cytokinesis, but also induced differentiation of Dami cells toward megakaryocytic lineage as indicated by expression of platelet-specific antigens in the model used in our work and by functional expression of glycoprotein IX, a megakaryocyte-specific gene and GTP-RO in previous investigations (Taniguchi et al., 1999b; Eibach et al., 2001). Moreover, Nocardazole and PMA induced phosphorylation of the proteins which are involved in translation signal transduction (e.g., S6K1, 4E-BP1, or eIF4E) and increased the expression of cyclins (cyclin D1 or cyclin D3) and cyclin-dependent kinase inhibitors (p21 or p27), which are involved in polyploidization as well. Thus, the model used in this work would most likely mimic the polyploidization in primary megakaryocytes and the data obtained in the model would give rise to crucial clues to elucidate mechanism of endomitosis in primary megakaryocytes.

Eukaryotic mRNAs contain a cap consisting of a modified guanosine residue at one end (the 5′ end). It has been shown recently that, during mitosis when bound to 14-3-3, eIF4B cannot bind to the 3′ cap. Consequently, cap-dependent translation is suppressed and only mRNA sequences with internal ribosome entry sites (IRES) domains can be translated. If mRNAs for cell-cycle regulator p58-PI3TSLR, can undergo translation in a cap-independent manner (Wilker et al., 2007). The translation of p27 was mediated via an IRES element in the 5′UTR (Kullmann et al., 2002). In our nocardazole-induced polyploid Dami cells, the expression of p27 increased. Therefore, further study needs to be done to clarify whether the phosphorylation of S6K1 at Thr421/Ser424 regulates the cap-dependent translation or the cap-independent translation.

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Literature Cited


