EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 3, 222-226, June 2003

Synergistic activation of p70S6 kinase associated with stem cell factor in MO7e cells

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Accepted 21 May 2003

Abbreviations: FRAP/mTOR, FKBP12-Rapamycin associated protein/mammalian target of Rapamycin; GM-CSF, granulocyte macrophage colony stimulating factor; JAK/STAT, Janus kinase/signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositol 3 kinase; p21, $p21^{cip1/waf1}$; p70S6K, p70S6 kinase; SCF, stem cell factor

Abstract

Stem cell factor (SCF) is an early-acting cytokine inducing proliferative synergy with other cytokines in hematopoietic cells. We earlier showed that p21 was synergistically induced in SCF synergy and the p44/42 MAPK pathway was essential for the transcriptional control of p21. SCF synergy accompanies protein synthesis. p70S6K implicated in translational control in many other systems has not been shown in SCF synergy induced system. GM-CSF dependent human cell line MO7e was stimulated with GM-CSF with SCF, and investigated activation of p70S6K by using phospho-specific antibody. A possible contribution of p70S6K to SCF synergy was examined by measuring p21 induction as a model system. p70S6K was slightly activated by GM-CSF alone and markedly activated by SCF alone. Combined stimulation with these two cytokines synergistically activated p70S6K resulting in persistent activation. Addition of the pathway-specific inhibitors for PI3K or FRAP/TOR, two upstream pathways of p70S6K resulted in abolishment of p70S6K phosphorylation and also significant reduction of p21 protein level. These data suggest that synergistically activated p70S6K by GM-CSF plus SCF involves, at least in part, protein translational control including regulation of p21 protein.

Keywords: hematopoietic progenitors, SCF synergy, p70S6K, translational control

Introduction

Environments surrounding cells in biological system are a milieu of many different factors, and the effects of these factors determine the fate of cells, such as growth, apoptosis, differentiation, and self-renewal. In the hematopoietic system, cytokines and chemokines are important regulators affecting cell's fate (Broxmeyer, 1999). The growth factor dependent cell line, MO7e, was derived from a patient with acute megakaryocytic leukemia, and has been widely used as a useful model system for hematopoietic progenitor cells (Avanzi *et al.*, 1988; Hendrie *et al.*, 1991). Stem cell factor (SCF: steel factor: SLF), an early acting cytokine, has remarkable capacity to synergize with other hematopoietic cytokines, and has been implicated in maintenance of hematopoiesis (Broxmeyer *et al.*, 1991). However, the mechanisms mediating these synergistic effects are still poorly understood. SCF does not alter the affinity or numbers of GM-CSF receptors (Hendrie *et al.*, 1991; Hallek *et al.*, 1994). Moreover, IL-3 and GM-CSF do not affect c-kit mRNA levels (Hu *et al.*, 1994). We previously reported that the protein level of cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ (p21) increased synergistically when MO7e cells were stimulated with SCF plus GM-CSF and that p21 induction was required for SCF synergistic responses (Mantel *et al.*, 1996). In order to better understand SCF synergy, we investigated intracellular signaling events after combined stimulation with GM-CSF plus SCF and evaluated molecular mechanisms involved in p21 synergy (Lee *et al.*, 2001; Lee and Broxmeyer, 2001). We found that only p44/42 MAPK (ERK1/2), among three different MAPK pathways, was synergistically activated by combined stimulation with GM-CSF plus SCF and it was essential for p21 induction at

the transcriptional level (Lee *et al.*, 2001). We also found that the PI3K/Akt pathway was activated in our system, although it was not synergistic and that induction of c-fos mRNA correlated well with ribosomal S6 kinase (RSK) activation, a downstream molecule of ERK1/2 and PI3K pathways (Lee and Broxmeyer, 2001).

Proliferative synergy of SCF plus GM-CSF accompanies protein synthesis, but regulation of protein synthesis has not yet been clearly defined. Recently, signaling pathways involved in translation control have become better understood (Dufner and Thomas, 1999). Growth factor-regulated signaling pathways downstream of phosphoinositol 3 kinase (PI3K) and FKBP12-Rapamycin associated protein/mammalian target of Rapamycin (FRAP/mTOR) are suggested to be involved in the regulation of translation initiation. p70/p85 S6Ks are two isoforms of the kinase that control the ribosomal protein S6 phosphorylation in response to mitogen, which are regulated by PI3K and FRAP/mTOR. p70/p85 S6K is activated by all mitogenic stimuli, including growth factors, cytokines, and phorbol esters, and plays an essential role in controlling the translational machinery (Chou and Blenis, 1995; Pullen and Thomas, 1997).

In this work, we investigated the contribution of the PI3K-p70S6K axis in the control of protein synthesis in SCF synergy. We found that p70S6K was synergistically activated and associated with p21 induction, which we previously implicated in SCF synergy.

Materials and Methods

Materials

PI3K inhibitor LY 294002, and p70S6K inhibitor Rapamycin were purchased from Calbiochem (La Jolla, CA). Dimethyl Sulfoxide (DMSO) and another PI3K inhibitor, Wortmannin, were purchased from Sigma (St. Louis, MO). MAP kinase inhibitor PD 98059 was obtained from Calbiochem.

Cell culture

Human growth factor dependent cell line MO7e was cultured in RPMI 1640 supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml of purified recombinant human GM-CSF (Immunex Corp, Seattle, WA). Before factor stimulation, the cells were incubated in RPMI 1640 supplemented with 1% bovine serum albumin (BSA) for 16 to 18 h without growth factors (factor starvation). Factor-starved cells were stimulated for various periods of time with saturating concentrations of either purified recombinant human GM-CSF (100 U/ml), or SCF (50 ng/ml; R&D Systems, Minneapolis, MN), or the combination of these two cytokines. When inhibitors were used, cells were preincubated for 1 h at 37°C before factor stimulation.

Cell lysate preparation and Western blotting

Cell lysate preparation and analysis by Western blotting with indicated antibodies were performed as previously described (Anzai *et al.*, 1999). Antibody to p21 was from Calbiochem. Antibodies to p70S6K and phospho-p70S6K (Ser389) were from Cell Signaling Technology (Beverly, MA).

RNase protection assay (RPA)

Total cellular RNA from MO7e cells stimulated with GM-CSF and/or SCF was isolated using MicroRNA Isolation kit (Stratagene, La Jolla, CA). For detection and quantitation of mRNA species modulated in MO7e cells, ribonuclease protection assay was performed as described previously (Lee *et al.*, 2001; Shin *et al.*, 2002). ^{32}P-UTP labeled RNA probe was prepared by *in vitro* transcription using Multi-probe template set hSTRESS-1 (Pharmingen, San Diego, CA) as a template. RNase-protected probes were purified, resolved on 5% denaturating polyacrylamide gels, and quantitated by autoradiography.

Results and Discussion

Synergistic activation of p70S6K in the combined stimulation of SCF plus GM-CSF

Proliferative synergy of SCF plus GM-CSF accompanies protein synthesis, but its regulation has not yet been clearly defined. Ribosomal p70S6K is known to play a critical role in translational control by phosphorylating ribosomal protein S6 in a 48S translation initiation complex (Chou and Blenis, 1995; Dufner and Thomas, 1999). Phosphorylation of S6 by p70S6K stimulates the translation of mRNAs with a 5' oligopyrimidine tract, typically encode components of the protein synthesis apparatus. p70S6K activation after stimulation of MO7e cells with GM-CSF plus SCF was monitored with the antibody specific to phospho-p70S6K (Ser389). Activated p70S6K has several phosphorylated serine residues, and among them phosphorylation at Ser 389 represents its enzymatic activity most well (Pullen and Thomas, 1997). p70S6K activation examined at different time points after stimulation showed that enzyme activity was correlated with its phosphorylation level. GM-CSF alone barely induced p70S6K activation whereas SCF induced much potent activation. Combined stimulation with GM-CSF plus SCF synergistically activated p70S6K resulting in its persistent activation (Figure 1A, clear at 1 h points).

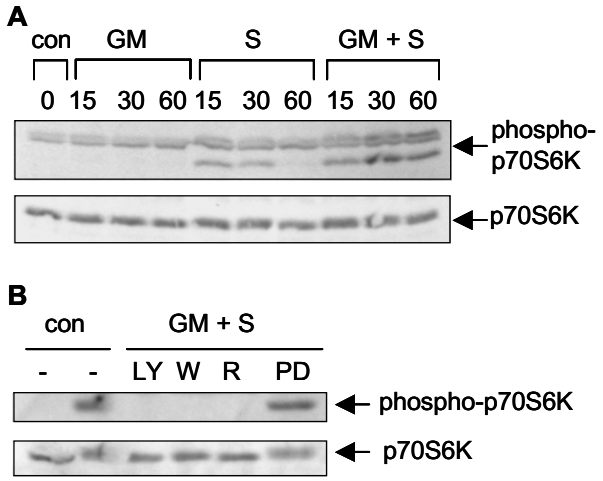

Figure 1. Synergistic activation of p70S6K after stimulation with GM-CSF and/or SCF; which was sensitive to PI3K inhibitors and Rapamycin. (A) MO7e cells were stimulated with 100 U/ml GM-CSF (GM) and/or 50 ng/ml SCF (S) for the indicated time periods. (B) MO7e cells were preincubated in the presence of DMSO vehicle (DM), Wortmannin (W; 100 nM), LY 294002 (LY; 30 μM), Rapamycin (R; 10 nM), or PD 98059 (PD; 50 μM) for 1 h and then stimulated with GM-CSF plus SCF for 30 min. Cell lysates were analyzed by SDS-PAGE and Western Blotting with phospho-specific antibody to p70S6K (Ser389). The membrane was stripped and reblotted with antibody recognizing p70S6K to show equal loading. These results are representative of two different experiments.

Such results indicate that SCF may be more important than GM-CSF in the process of translational regulation.

Activation of p70S6K is sensitive to PI3K inhibitors as well as TOR inhibitor Rapamycin

PI3K and FRAP/mTOR are known to be two upstream pathways of p70S6K (Dufner and Thomas, 1999). To determine the participation of the p70S6 kinase pathway in SCF synergy, MO7e cells were pretreated with specific inhibitors for these pathways and then stimulated the cells with GM-CSF plus SCF. PI3K inhibitors Wortmannin (100 nM) or LY 294002 (30 μM) and FRAP/mTOR inhibitor Rapamycin (10 nM) were used as inhibitors. Specific inhibitor for the MEK1/MAPK pathway, PD 98059, was used as a control. Based on the phosphorylation level of p70S6K, both PI3K and TOR inhibitors abolished activation of p70S6K induced by combined stimulation with GM-CSF plus SCF. In contrast, PD 98059 had no effect (Figure 1B). This suggests that p70S6K activation through SCF synergy is both PI3K-dependent and FRAP/mTOR-dependent.

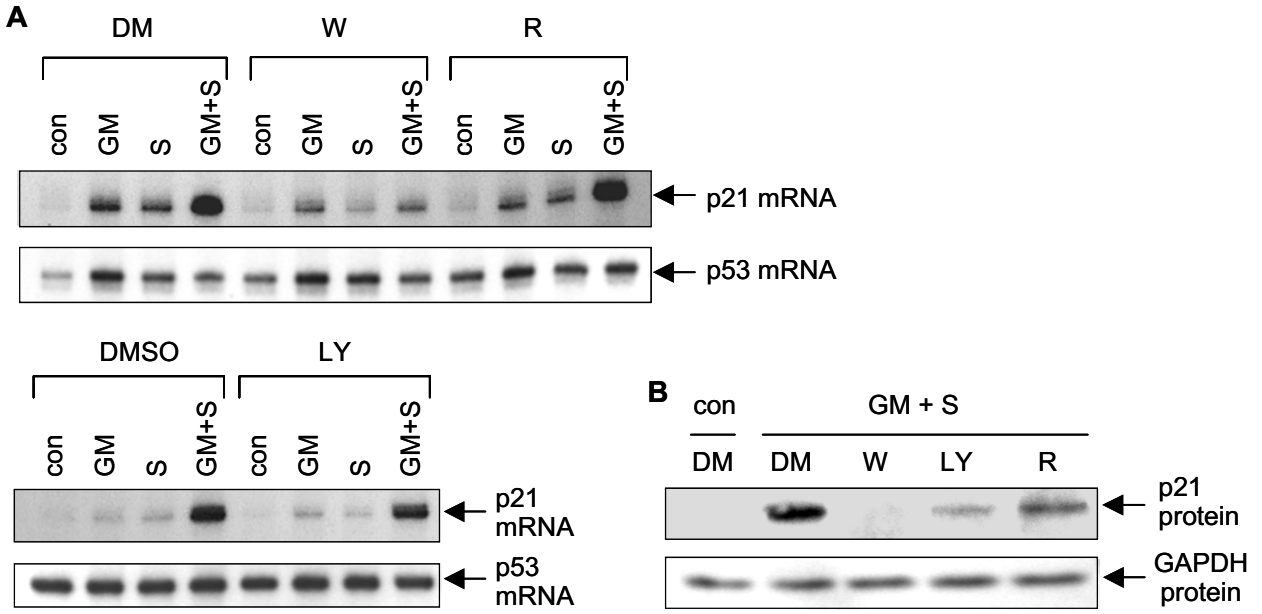

Figure 2. Regulation of p21 not at the mRNA level but at the protein level by pretreatment of cells with PI3 kinase inhibitors or Rapamycin. MO7e cells were preincubated in the presence of vehicle control DMSO (DM), Wortmannin (W), LY 294002 (LY), or Rapamycin (R) for 1 h and then stimulated with 100 U/ml GM-CSF (GM), 50 ng/ml SCF (S), or with combinations of these cytokines (GM+S) for 2 h. (A) mRNA level of p21 was investigated by RNase protection assay. p53 mRNA level was shown as a loading control. (B) Protein level of p21 was examined by Western blotting using antibody to p21. GAPDH was used as a loading control. These experiments were performed twice yielding similar results.

Activation of p70S6K is necessary for full induction of p21 in SCF synergy

To determine the contribution of the p70S6K pathway in synergistic induction of p21, mRNA and protein levels of p21 were examined following the cells induced by GM-CSF plus SCF in the presence of specific pathway inhibitors for PI3K and FRAP/TOR.

Synergistic induction of p21 was reduced dramatically by the pretreatment with Wortmannin (Figure 2A), but the effect of LY 294002 (30 μM) was not significant at the mRNA level (Figure 2A: less than 10% reduction). To clarify the discrepancy between effects of the two PI3K inhibitors, their effects on p44/42 MAP kinase activation were measured. Wortmannin did reduce MAP kinase activation, but LY 294002 did not (data not shown). Therefore, it is likely that abolishment of p21 induction in the presence of Wortmannin might be due to the inhibition of p44/42 MAP kinase, which is very essential for p21 induction in this proliferative synergy (Lee *et al.*, 2001). The mRNA level of p21 was not changed in the presence of Rapamycin (Figure 2A). Therefore, PI3K and FRAP/mTOR do not appear to be involved in transcriptional regulation of p21 after stimulation with GM-CSF plus SCF.

The protein level of p21 was also examined in the presence of these inhibitors. Treatment with Wortmannin abolished induction of p21 protein by GM-CSF plus SCF presumably because of p44/42 MAPK inhibition. In contrast with mRNA level, p21 protein level was significantly reduced in the presence of LY 294002 and Rapamycin (Figure 2B). Together, these data suggest that the PI3K and FRAP/mTOR pathways appear to be involved in p21 synergy at the protein level possibly by activating p70S6K, but not at the mRNA level.

In MO7e cells, SCF induced prominent activation of PI3K leading to high levels of Akt phosphorylation, which contributes to the induction of immediate early gene c-fos (Lee and Broxmeyer, 2001). These results suggest another possible role of PI3K: regulating protein synthesis and contributing to synergistic induction of p21 at the protein level.

Multiple signaling involved in SCF synergy

SCF is an early acting co-stimulatory cytokine, and its synergistic actions with other cytokines are crucial to maintain the homeostasis of hematopoietic system (Broxmeyer *et al.*, 1991). We previously investigated molecular mechanisms involved in SCF synergy and reported transcriptional up-regulation of c-fos, jun-B, and p21 (Horie and Broxmeyer, 1993; Lee *et al.*, 2001; Lee and Broxmeyer, 2001). The examination of mRNA levels of several other genes showed that cyclin G2 was synergistically reduced by combined stimulation

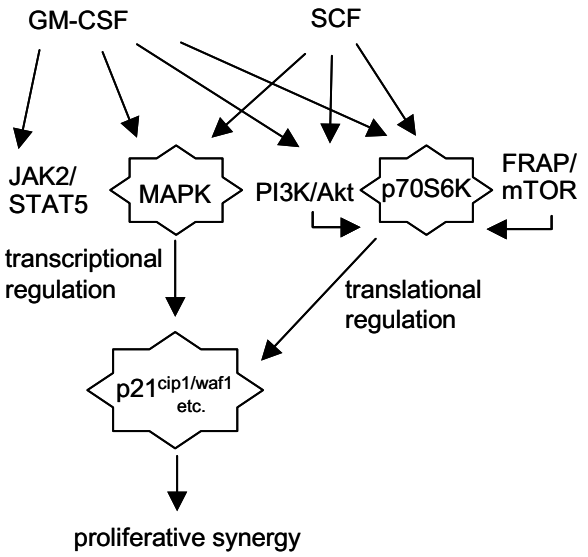

Figure 3. Schematic view of signaling pathways involved in SCF synergy. Combined stimulation with GM-CSF and SCF in MO7e cells activates multiple signaling pathways, and their combined cross talk may result in p21 synergy and contribute to SCF synergy.

with GM-CSF plus SCF (unpublished data). Cyclin G2 was reversely correlated with cell proliferation (Horne *et al.*, 1996; Horne *et al.*, 1997) and synergistic down-regulation may be one important mechanism. Here, we suggest translational controls involved in this proliferative synergy by focusing on p70S6K activation with a model protein, p21. As shown schematically in Figure 3, SCF synergy accompanies activation of multiple signaling cascades including MAPK, PI3K, JAK/STAT, and p70S6K. MAPK appears to be mainly involved in transcriptional control, while PI3K and p70S6K may contribute to translational control. As a consequence, downstream genes are up-regulated or down-regulated at the mRNA or protein level to set an overall cellular state proper for synergistic proliferation. Although the extent of each pathway's contribution and exact regulation mode are still unclear, the above studies should help in directing us to better understand the complex biological phenomena of synergistic responses to multiple growth factors, including that of SCF.

Acknowledgement

This work was supported by U.S. Public Health Service Grants R01 HL56416, R01 HL67384, and R01 DK53674 from the US National Institutes of Health to Hal E. Broxmeyer. This research was also partly supported by a grant (M102KL010001-02K1201-00410) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

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