SP600125 Inhibits Cap-dependent Translation Independently of the c-Jun N-terminal Kinase Pathway

Masatoshi Ito¹,³, Hiroshi Kitamura¹,⁴*, Chisato Kikuguchi¹, Koji Hase², Hiroshi Ohno²,³, and Osamu Ohara¹,⁵

Laboratories for ¹Immunogenomics and ²Epithelial Immunobiology, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan, ³Department of Supramolecular Biology, Graduate School of Nanobioscience, Yokohama City University, Yokohama 230-0045, Japan, ⁴Department of Comparative and Experimental Medicine, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan, ⁵Department of Human Genome Research, Kazusa DNA Research Institute, Kisarazu 292-0818, Japan

ABSTRACT. We investigated the effects of SP600125 (formerly called c-Jun N-terminal kinase (JNK) inhibitor II) on translation using cultured mouse cells. SP600125 (50 µM) treatment rapidly repressed overall protein synthesis, accompanied by a reduction in the mRNAs for housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase in the polysomal fraction. SP600125 decreased polysomes with a concomitant increase in free ribosomal subunits in the cytoplasm, suggesting that global translation was inhibited at the initiation step. A reporter analysis using exogenous mRNAs showed that SP600125 inhibited cap-dependent but not internal ribosome entry site-dependent translation. SP600125 significantly attenuated phosphorylation of components in the mTOR pathway, which is responsible for cap-dependent translation. In contrast to SP600125, short hairpin RNAs for JNK1 and JNK2 failed to affect overall protein synthesis. Collectively, SP600125 inhibits cap-dependent translation, independent of the JNK pathway.

Key words: SP600125/cap-dependent translation/polysome/c-Jun N-terminal kinase

Introduction

Translation of mRNAs into proteins is one of the most fundamental biochemical processes in gene expression regulation. In eukaryotes, translation is predominantly controlled at the initiation step, during which a 40S ribosome subunit is recruited to an mRNA via two different mechanisms (Hershey, 1991). The translation of most (approximately 97%) eukaryotic mRNAs depends on a 7-methylguanosine (m7GpppN, where N is any nucleotide) cap-structure at their 5' termini, while that of the remaining (approximately 3%) mRNAs relies on an internal ribosome entry site (IRES) (Merrick, 2004). Accumulating evidence indicates that the mammalian target of rapamycin (mTOR) plays a critical role in the cap-dependent translation (Hershey and Merrick, 2000; Brown and Schreiber, 1996). mTOR directly phosphorylates two translational regulators, namely 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (S6K), and subsequently activates translation initiation factors such as eukaryotic initiation factor (eIF) 4B and eIF4E (Ma and Blenis, 2009; Mamane et al., 2006).

SP600125 {SP, anthra[1,9-cd]pyrazol-6(2H)one} is a small chemical compound originally identified during anticancer drug screening (Showalter et al., 1987). Since Bennett et al. reported that SP strongly inhibited c-Jun N-terminal kinase (JNK) activity (Bennett et al., 2001), this compound has been recognized as a JNK inhibitor II to
decipher the roles of the JNK pathway (Huang et al., 2003). However, Bain et al. demonstrated by in vitro experiments that SP directly inhibited activities of various protein kinases with a similar or greater potency than JNKs (Bain et al., 2003). SP especially inhibits the kinase activity of S6K and phosphoinositide-dependent kinase 1 (PDK1) that are proposed to modulate protein synthesis (Pullen et al., 1998). Despite these pioneer studies, the effects of SP on translation have not yet been explored. In this study, we demonstrate that SP inhibits cap-dependent translation in a JNK-independent manner.

**Materials and Methods**

**Cell culture and reagents**

Mouse macrophage cell line J774.1 and fibroblast cell line Swiss 3T3 were obtained from the RIKEN Bioresource Center (Tsukuba, Japan) and maintained according to a standard protocol (Freshney, 2000). SP was purchased from Calbiochem (La Jolla, CA, USA).

**Subcellular fractionation of sucrose density gradients and polysome analysis**

Polyosomal and non-polyosomal fractions were prepared using a 15–50% sucrose density gradient as previously described with minor modifications (Kitamura et al., 2008): we collected fractions 1–11 and 12–20 of 20 total fractions as the non-polyosomal and polysomal fractions, respectively. Sucrose sedimentation analysis was performed by continuously monitoring the sucrose density gradients at 260 nm using a BioRad EM-1 monitor (Hercules, CA, USA) connected to a Piston Gradient Fractionator (BioComp Instruments, New Brunswick, Canada). The spectrum pattern was scanned, and dimensions of polysome areas were quantified using a Multi Gauge version 3.0 software package (Fujifilm, Tokyo, Japan).

**Northern blot analysis**

RNA extraction and Northern blot analysis were conducted as previously described (Kitamura et al., 2002). Membranes were hybridized with [α-32P]dCTP-labeled cDNA probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2 microglobulin (B2M), hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) and peptidylprolyl isomerase A (PPIA), and then scanned using a BAS2000 Bioimage Analyzer (Fujifilm).

**Western blot analysis**

Western blot analysis was performed as previously described (Kitamura et al., 2008). Membranes were incubated with anti-phospho-PDK1 (Ser241), phospho-AKT (Ser473), AKT, phospho-mTOR (Ser2448), mTOR, 4EBP1, phospho-S6K (Thr389), phospho-eIF4B (Ser422), eIF4B, phospho-JNK1/2 (Thr183/Tyr185), JNK1/2, phospho-c-Jun (Ser63), c-Jun (Cell Signaling Technology, Beverly, MA, USA), GAPDH, S6K, and JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Primary antibody immunoreactivity was visualized with an ECL plus kit (GE Healthcare, Piscataway, NJ, USA) and then scanned using an LAS1000 Bioimage Analyzer (Fujifilm).

**Metabolic labeling of newly synthesized proteins**

Newly synthesized proteins were metabolically labeled as previously described (Kitamura et al., 2008). To analyze GAPDH protein synthesis, 500 μg of cytoplasmic proteins, extracted with a buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM EDTA, 1% Nonidet P40 and a complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), were immunoprecipitated with an anti-GAPDH antibody (Santa Cruz Biotechnology) and then subjected to Western blot analysis.

**Luciferase reporter assay using cap and IRES reporter mRNAs**

Firefly luciferase cDNA was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), yielding pT7-luc. The encephalomyocarditis virus-derived IRES was then cloned into pT7-luc (pT7-IRES-luc). After linearization with BamHI, both pT7-luc and pT7-IRES-luc plasmids were used as a template for in vitro transcription with an mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) in the presence of m’GpppG and adenosyl(5’)ppp(5’) guanosine (AppG) (New England Biolabs, Beverly, MA, USA), respectively. After the addition of a poly(A)+ tail, the synthesized mRNAs were transfected into Swiss 3T3 cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in the presence or absence of 50 μM SP for 1 h. At 3 h and 6 h post transfection, the chemiluminescent signal was measured using a Fusion microplate reader (Perkin Elmer, Waltham, MA, USA). In parallel, the transfection efficiency of the reporter mRNAs was evaluated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) using a LightCycler480 (Roche, Sussex, UK). Primer sequences were 5’-GTTGCAAAACGCTTCCATCT-3’ (forward) and 5’-CCGGTATCCAGATCCACAAC-3’ (reverse).

** Knockdown of JNK1 and JNK2 with short hairpin RNAs (shRNAs)**

Lentiviruses expressing pre-designed shRNAs for JNK1 and JNK2, as well as a non-targeting shRNA, were obtained from Sigma-Aldrich (St. Louis, MO, USA). J774.1 cells infected with each virus were cloned in a selection medium containing 1 μg/mL of puromycin for two weeks. Target sequences of shRNAs used in this study were as follows; non-target, 5’-CAACAAGATGAGACACCAAA-3’; JNK1, 5’-CGTCTGTCAATGACATGTCTT-3’; JNK2, 5’-GCTATGATTTGATCCAGACA-3’.
Suppression of Global Translation by SP600125

Statistical analysis

Data were expressed as the means±SD. Statistical analysis was performed by one-way analysis of variance followed by the Bonferroni-type post hoc test.

Results

SP600125 inhibits translation in a global manner

A previous *in vitro* study indicated that SP directly inhibited protein kinases that potentially control translation (Bain *et al.*, 2003). Thus, we first evaluated whether SP would change overall protein synthesis in cultured cells. We incubated J774.1 cells in the presence or absence of different concentration of SP (10–100 μM) for 1 h, added $[^{35}S]methionine$ and cysteine (Met/Cys) for 30 min to metabolically radiolabel these cells, and then measured the radioactivity of the total protein pool (Fig. 1A). Although the vehicle control (DMSO) did not change the total radioactivity of the pool, SP significantly decreased the activity in a dose-dependent manner. Compared with the DMSO-treated cells, the SP-treated cells showed 24±1%, 42±4%, 52±2%, and 54±2% reductions in the radioactivity at 10 μM, 30 μM, 50 μM, and 100 μM of SP, respectively. Similar to J774.1 cells, 50 μM of SP also caused the maximum reduction of radiolabeled proteins in Swiss3T3 cells (Fig. 1B). Thus, SP decreased overall protein synthesis in cultured cells. SDS-polyacrylamide gel electrophoresis analysis further showed that SP evenly decreased all detectable protein bands labeled with $[^{35}S]Met/Cys$ without affecting Coomassie brilliant blue staining patterns (Fig. 1C). This suggests that SP potentially inhibits global translation.

To confirm the inhibitory action of SP on translation, we monitored the effects of SP on the translation of four...
housekeeping genes, namely GAPDH, B2M, HPRT1 and PPIA, whose mRNA abundances are insusceptible to external stimuli (Champelovier et al., 2010). We treated J774.1 cells and Swiss 3T3 cells with SP (50 μM) or DMSO for 1 h and then measured mRNA levels of GAPDH, B2M, HPRT1 and PPIA in the total cytoplasmic, polysomal and non-polysomal fractions (Fig. 1D). Although SP failed to modulate mRNA levels of the genes in the total cytoplasmic fraction, SP caused a concomitant decrease and increase of the mRNAs in the polysomal and non-polysomal fractions, respectively. To further evaluate the effects of SP on translation, we performed immunoprecipitation of GAPDH protein using metabolically labeled cells with or without the SP treatment. SP treatment resulted in approximately 40% decrease in the radiolabeled GAPDH protein, while SP did not influence the total amount of GAPDH protein in the precipitate (Fig. 1E). Taken together, these results indicated that SP significantly inhibits translation of the tested housekeeping genes. This observation supports the idea that SP is an inhibitor of global translation.

**SP600125 suppresses cap-dependent translational initiation**

We next determined the steps in translation that are inhibited by SP using sucrose sedimentation analysis (Fig. 2A). Compared with the untreated cells, DMSO-treated cells exhibited only marginal changes in the spectrum patterns that consisted of non-polysomal peaks corresponding to 40S, 60S and 80S, and some polysomal peaks. In contrast, SP elevated the non-polysomal peaks and lowered the polysomal peaks. The areas corresponding to polysome decreased to approximately 72% (J774.1 cells) and approximately 71% (Swiss 3T3 cells) by the SP treatment. Since similar changes of polysomal and non-polysomal peaks have been observed in the cells defective in translational initiation (Soni et al., 2008), SP is likely to inhibit translational initiation.

To assess the effect of SP on two distinct initiation mechanisms, namely cap-dependent and IRES-dependent mechanisms, we conducted a reporter assay using two types of luciferase mRNAs that contained either a cap or IRES
structure. In this experiment, we only analyzed Swiss 3T3 cells due to the inability to efficiently transfect these reporter mRNAs into J774.1 cells (data not shown). In the cap-reporter transduced cells, SP decreased the luciferase activity to 44±13% and 40±11% at 3 h and 6 h post transfection, respectively (Fig. 2B). In contrast, SP did not influence the activity in the IRES-reporter transduced cells. Therefore, SP appears to inhibit the translation of cap mRNAs, but not IRES bearing mRNAs.

Since mTOR signaling is known to be crucial for the initiation step of cap-dependent translation (Hershey and Merrick, 2000; Brown and Schreiber, 1996), we next examined the effects of SP on the phosphorylation status of the mTOR signaling components. In addition to mTOR itself, upstream and downstream molecules of the mTOR pathway, namely PDK1, AKT, 4EBP1, S6K and eIF4B, were slightly phosphorylated in J774.1 cells and Swiss 3T3 cells under normal growth conditions (Fig. 2C). SP attenuated the phosphorylation of these molecules without affecting their total protein levels, although the compound did not affect PDK1 phosphorylation. Thus, SP inhibits the initiation step of cap-dependent translation, potentially by inhibiting the mTOR signaling.

**Gene knockdown (KD) of JNKs does not affect overall protein synthesis**

Since SP (50 μM) inhibited phosphorylation of JNKs in J774.1 cells and Swiss 3T3 cells (Fig. 3A), translational inhibition by SP might be attributed to JNK inactivation. While the JNK family consists of JNK1, JNK2 and JNK3 in the mammalian genome, JNK3 is not expressed in both J774.1 cells and Swiss 3T3 cells (data not shown). Thus, we examined the roles of JNK1 and JNK2 in translation. We established two respective J774.1-derived clones that stably express JNK1-targeting, JNK2-targeting, or non-targeting shRNAs (Fig. 3B). Compared with the non-targeting shRNA expressing clones, the JNK1-shRNA expressing clones considerably decreased JNK1 protein levels (sum of abundance of p46 and p54 JNK1 proteins, 47±9% (clone 1) and 44±2% (clone 2)) without affecting JNK2 protein levels. JNK2 protein expression in the JNK2-shRNA expressing clones was reduced to 23±12% (clone 1) and 27±17% (clone 2) without affecting JNK1 protein levels. In a previous study, Jnk1-deficient cells showed a significant reduction in c-Jun phosphorylation, whereas a Jnk2-deficiency promoted the phosphorylation (Sabapathy et al., 2004). Likewise, JNK1-KD and JNK2-KD cells showed a decrease and increase, respectively, in c-Jun phosphorylation (Fig. 3C), suggesting that the JNK shRNAs caused a sufficient decrease in the kinase activity of corresponding JNKs. However, neither the JNK1-KD nor the JNK2-KD clones exhibited any significant changes in the total mass of newly synthesized proteins [JNK1-KD: 102±2% (clone 1, *P*=0.9564) and 93±7% (clone 2, *P*=0.1604), JNK2-KD: 91±2% (clone 1, *P*=0.1384) and 89±5% (clone 2, *P*=0.0704)] (Fig. 3D). Therefore, JNKs are unlikely to be involved in the translational inhibition caused by SP treatment.
Discussion

Although SP inhibits the activities of various protein kinases proposed to be involved in translational control (Bain et al., 2003), the effects of SP on translation have not yet been explored. In this study, we demonstrated for the first time that short-term (1 h) treatment of SP reduced overall protein synthesis. We also observed that SP consistently inhibited the synthesis of all detectable proteins in an electrophoretic analysis. Moreover, SP inhibits the translation of housekeeping genes whose expression is insensitive to external stimuli. Thus, SP is likely to affect the fundamental machinery involved in translation. In agreement with this idea, our preliminary experiment reveals that SP also inhibits the translation of genes participating in basic cellular functions, such as glycolysis (e.g. 6-phosphogluconolactonase) and mitochondrial oxidative phosphorylation (e.g. NADH dehydrogenase 1 β subcomplex 11 and mitochondrial ATP synthase δ subunit).

In this study, SP augmented abundance of free 40S and 60S ribosomal subunits in the cytoplasm, accompanied by a decrease in polyosomes. This observation is similar to the suppression of cap-dependent translation observed at the initiation step (Soni et al., 2008). In agreement, reporter analysis using exogenous mRNA clearly indicates that SP inhibits cap-dependent translation but not IRES-dependent translation. SP also inhibited mTOR signaling, which is required for cap-dependent translation at the initiation step (Hershey and Merrick, 2000; Brown and Schreiber, 1996). Since almost all (approximately 97%) proteins employ cap-dependent translation, inhibition of such translation might contribute to a reduction in overall protein synthesis caused by SP treatment. While SP treatment clearly decreased global translation, neither JNK1-KD nor JNK2-KD caused translational inhibition. Therefore, JNKs do not seem to be responsible for the translational inhibition caused by SP treatment. A previous in vitro study demonstrated that SP directly interrupted catalytic reactions of S6K and PDK1, both of which are potentially involved in the mTOR pathway (Brown and Schreiber, 1996). In this study, phosphorylation of S6K, but not PDK1, clearly declined in cultured cells after SP treatment, indicating direct inhibition of S6K might contribute to the translational inhibition. In addition, AKT and mTOR (upstream molecules of S6K), and 4EBP1 (a molecule without causal connection to S6K), underwent remarkable dephosphorylation after SP treatment. Thus, an unknown target molecule(s) potentially participates in SP-elicited translational inhibition.

SP (50 μM) caused remarkable suppression of global translation in cultured cells in our study, which was in accordance with its severe effects on cell viability in vitro (Moon et al., 2008; Du et al., 2004; Ito and Kitamura, unpublished data). In contrast, previous in vivo studies demonstrated 10 mg/kg of SP failed to cause severe health defects for several days in mice (Kim et al., 2009; Wang et al., 2004). Since an approximate calculation indicated that the in vivo doses are comparable to our in vitro dose {10 mg/kg (an effective dose of SP in vivo)×0.04 g (body weight of a mouse)×1/220.2 mol/g (molecular weight of SP)×28 ml (moisture in a mouse)=64.9 μM}, there is an apparent discrepancy in the observed harmful effects between in vivo and in vitro studies. One of the reasons for this discrepancy might be attributed to each cell possessing a variable sensitivity to SP. Although 10 mg/kg of SP completely suppressed JNK activation in vivo, this dose may not be sufficient to affect global translation in various cell types. This idea is supported by the finding that 50 μM of SP did not change phosphorylation of AKT, a pivotal kinase for cap-dependent translation, in HEK293 cells, while the dose clearly decreased the phosphorylation in J774.1 and Swiss 3T3 cells (Fig. 2C and unpublished data). Thus, certain cell types that directly affect health conditions might be relatively resistant to SP-elicited translational inhibition. Another possibility is that variation in the significance of translational control may also account for the difference between in vivo and in vitro data. For example, translation inhibitors, such as cycloheximide and anisomycin, caused cell death in macrophages even at low doses (approximately 50% of cell death, 0.3 μg/ml, 24 h treatment), whereas higher doses (approximately 30% of cell death, 30 μg/ml, 24 h treatment) is required to cause cell death of smooth muscle cells (Croons et al., 2009, 2007). Therefore, approximately 50% inhibition of translation might be insufficient to cause health defects in mice because cells resistant to translational suppression still maintain a normal function.

In conclusion, our results demonstrated that SP inhibits cap-dependent translation at the initiation step and thereby represses global translation, and that JNK is unlikely to be involved in the translational inhibition resulting from SP treatment.

Acknowledgments. The authors thank Drs. Bob Meek, Jun Okabe, Yoshinori Shimamoto, Mai Shirasaki-Yamagishi and Yoshitaka Shirasaki, and Michael Scott and Atsushi Hijikata for critically reading the manuscript. We also thank Tomoko Yuasa, Michiyto Takayama, and Saori Hayashi for technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 21790334 and 17780225) and the Takeda Science Foundation.

References

Suppression of Global Translation by SP600125


(Received for publication, November 29, 2010, accepted, December 25, 2010 and published online, January 20, 2011)