MEK-ERK is involved in SUMO-1 foci formation on apoptosis

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Small ubiquitin-related modifier (SUMO) modification appears to regulate the activity, intracellular localization, and stability of the targeted proteins. To explore the relationship among sumoylation, antitumor reagent, and apoptosis, we treated green fluorescence protein (GFP)-SUMO-1-overexpressed K562 cells (K562/GFP-SUMO-1) with mitoxantrone (MIT) as an antitumor reagent. By the treatment with MIT, GFP-SUMO-1 formed foci in nuclei. While by the treatment with a tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), GFP-SUMO-1 located homogeneously in nuclei. When K562/GFP-SUMO-1 cells were treated with TPA plus MIT, GFP-SUMO-1 foci became larger and apoptosis was induced more than with MIT alone. The apoptosis induced by TPA plus MIT was prevented by blockage of GFP-SUMO-1 foci by small interfering RNA (siRNA) against SUMO-1. The formation of GFP-SUMO-1 foci was reduced by a MEK inhibitor U0126 or a nuclear export inhibitor leptomycin B, and endogenous SUMO-1 foci were reduced in K562 cells expressing the dominant-negative MEK1 mutant. These results suggest that the formation of SUMO-1 foci is regulated by the MEK-ERK pathway and may induce apoptosis. (*Cancer Sci* 2007; 98: 569–576)

Small ubiquitin-related modifier (SUMO)-1, which is structurally related to ubiquitin, is a protein of 101 amino acid residues and a molecular mass of about 12 kDa. The available data currently implicate SUMO protein in the regulation of protein–protein interactions, subcellular nuclear localization, protein–DNA interactions, and enzymatic activity, and there is also evidence that SUMO protein can act as an antagonist of ubiquitin.[1–4]

The promyelocytic leukemia protein (PML) gene encodes a putative tumor suppressor gene involved in the control of apoptosis. PML nuclear bodies (NBs), which are formed by intact sumoylated PML,[5] have a variety of functions, including tumor suppression,[6] transcriptional regulation[7] and DNA repair.[8] Many proteins, such as nuclear dot protein Sp100, transcriptional regulator CREB-binding protein (CBP), and death domain-associated protein (Daxx), have been detected in these structures.[9] Many NB-associated proteins also appear to be covalently modified by SUMO-1. Disruption of PML NBs causes diseases related to tumorigenesis and resistance to their therapies. It is remained the question that how PML NBs are coupled to various apoptosis pathways.

Mitoxantrone (MIT) is a topoisomerase II inihibitor, which may act by interfering with the religating activity of the enzyme to DNA double-strand breaks.[10] Topoisomerases are also substrates of sumoylation and predominantly localized in the nucleolus.[11–14] However, it is not clear what the relationship is between the change of distribution of SUMO-1 and apoptosis induced by topoisomerase inhibitor, and whether the signal pathway influences the change of distribution of SUMO-1.

12-O-tetradecanoylphorbol-13-acetate (TPA) has been reported to induce megakaryocytic differentiation of K562 cells.[15,16] The sustained activation of the mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) pathway by TPA is required for megakaryocytic differentiation of K562 cells.[17] The distribution of SUMO-1 on TPA-induced megakaryocytic differentiation of K562 cells has not been previously reported.

Here, we examined the association between the intracellular distribution of SUMO-1 and apoptosis by treatment with or without an antitumor reagent MIT, in the presence or absence of TPA using GFP-SUMO-1-overexpressing K562 cells (K562/GFP-SUMO-1). Moreover, the MEK-ERK pathway is related to the formation of SUMO-1 foci.

Materials and Methods

Reagents. TPA, phorbol 12, 13-dibutyrate (PDBu) and U0126 were purchased from Sigma (St Louis, MO, US). MIT was obtained from Lederle (Tokyo, Japan). Leptomycin B (LMB) was kindly given by Yoshida M, Chemical Genetics Laboratory, RIKEN.

Plasmid construction of GFP-SUMO-1 and transfection. GFP-SUMO-1 was constructed as previously reported.[4] After linealization of this vector by ApaL I, 5 μg of the DNA was introduced into K562 cells by electroporation. After electroporation, the cells were selected by 400 μg/mL of G418, and then cloned in methylcellulose.

SiRNA for SUMO-1 and transfection. To generate double-stranded RNA for SUMO-1, the DNA sequence, corresponding to nucleic acids 1–130 in the SUMO-1 cDNA, was amplified by PCR. RNA transcription was then performed with this DNA template to generate sense and antisense single-stranded RNA. After production of double-stranded RNA, a reaction with the Dicer enzyme was carried out using a BLOCK-iT Dicer RNAi kit (Invitrogen) as described previously.[18] The siRNA (final concentration 50 nM) was transfected into K562/GFP-SUMO-1 cells using Oligofectamine (Invitrogen Carlsbad, CA, US) and the cells were incubated at 37°C for 48 h.

MEK1 dominant-negative cells. For construction of plasmid pTER2hyg-MEK1 K97A mutant, pCMV HA human MEK1 K97A DNA fragment encoding the full-length protein, which was kindly supplied by Goto Y, Institute of Molecular and Cellular Biosciences, University of Tokyo, was amplified by PCR. The PCR product was then inserted into the pTER2hyg vector. For the stable transfection, the regulator plasmid pTet-on and pTRE2hyg-MEK1 K94A mutant were transfected into K562 cells by using Tet-off™ and Tet-on™ Gene Expression Systems (Clontech, CA, US) according to the manufacture's instructions. Expression of MEK1 K97A mutant in the cell line was induced by incubation with 10 μg/mL doxycycline for 16 h, which is a tetracycline analog.

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Cell culture, preparation of protein extracts, and Western blotting. Wild-type K562 cells were cultured in RPMI-1640 (Gibco) containing 10% FBS (Gibco) at 37°C in 5% CO_2. K562/GFP-SUMO-1 cells were incubated in RPMI-1640 containing 400 μg/mL of G418.

After the treatment of antitumor reagent, cells were washed with PBS and collected by centrifugation. Pellets were boiled for 5 min in SDS sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 6% β-mercaptoethanol, and 0.01% bromophenol blue). The samples of equal amount of total protein were electrophoresed on SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and incubated with the specific antibodies overnight at 4°C. The membranes were incubated in the appropriate HRP-coupled secondary antibodies for 1 h at room temperature, followed by enhanced chemiluminescence detection of the proteins with ECL reagent (Amersham-Pharmacia Biotech, Piscataway, NJ, US). The antibodies were obtained from different sources as follows: mouse monoclonal anti-SUMO-1 (D-11) antibody from Santa Cruz Biotechnology, Inc. CA, US; rabbit polyclonal anti-ERK1/2 and rabbit polyclonal antiphospho-ERK1/2 antibodies from Cell Signaling (Beverly, MA, US).

TUNEL staining. The TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using the *In Situ* Cell Death Detection Kit, TMR-red (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Immunofluorescence staining and DNA staining. The cells were fixed with 4% paraformaldehide in PBS for 30 min at room temperature, permeabilized with permeabilization buffer (0.01% FBS and 0.1% TritonX-100 in PBS) for 20 min at room temperature. Endogenous SUMO-1 protein was immunostained with mouse monoclonal anti-SUMO-1 antibody diluted to 1 μg/mL in permeabilization buffer for 1 h at room temperature. After washing in PBS, 4 μg/mL (in permeabilization buffer) of Alexa Fluor 488-conjugated antimouse IgG antibody (Molecular Probes, Eugene, OR, US) was used for secondary antibody. For DNA staining, samples were incubated with 1 μM TOPRO-3 (Molecular Probes) or 5 μM Hoechst 33258 (Dojindo, Kumamoro, Japan) in PBS for 10 min at room temperature. Immunofluorescence microscopic analysis was performed by FV1000 (Olympus) confocal attached to microscope (model IX81; Olympus) using 60x oil immersion lens, and images were processed using FV10-ASW.

Results

Characteristics of expression of GFP-SUMO-1 in the treatment of MIT with or without TPA. To analyze the relationship between subcellular localization of SUMO-1 and exposure of antitumor reagent, we transfected SUMO-1 fused to GFP into K562 cells (K562/GFP-SUMO-1) and the cells were treated with or without an antitumor reagent MIT (0.5 μg/mL), in the presence or absence of a tumor promoter TPA (2 nM), for 24 h followed by TUNEL staining (Fig. 1). The intracellular localization of GFP-SUMO-1 was analyzed by laser scanning confocal fluorescence microscopy (Fig. 1a). GFP-SUMO-1 located homogeneously in nuclei by treatment with TPA (Fig. 1a, panel b), whereas GFP-SUMO-1 formed foci in nuclei by treatment with MIT (Fig. 1a, panel c). Interestingly, in the case of treatment with TPA plus MIT, the GFP-SUMO-1 foci became larger than with MIT alone (Fig. 1a, panels c and d).

Relationship between the formation of GFP-SUMO-1 foci and sensitivity to apoptosis. To investigate the relationship between the expression patterns of GFP-SUMO-1 and apoptosis induced by MIT, we treated wild-type K562 cells and K562/GFP-SUMO-1 cells as described in Fig. 1a and counted TUNEL-positive cells (Fig. 1b). When K562/GFP-SUMO-1 cells were treated with TPA, the percentage of TUNEL-positive cells was 2.2 ± 0.3%. On the other hand, when the cells were treated with MIT, the percentage of TUNEL-positive cells was 4.4 ± 0.7%. In the case

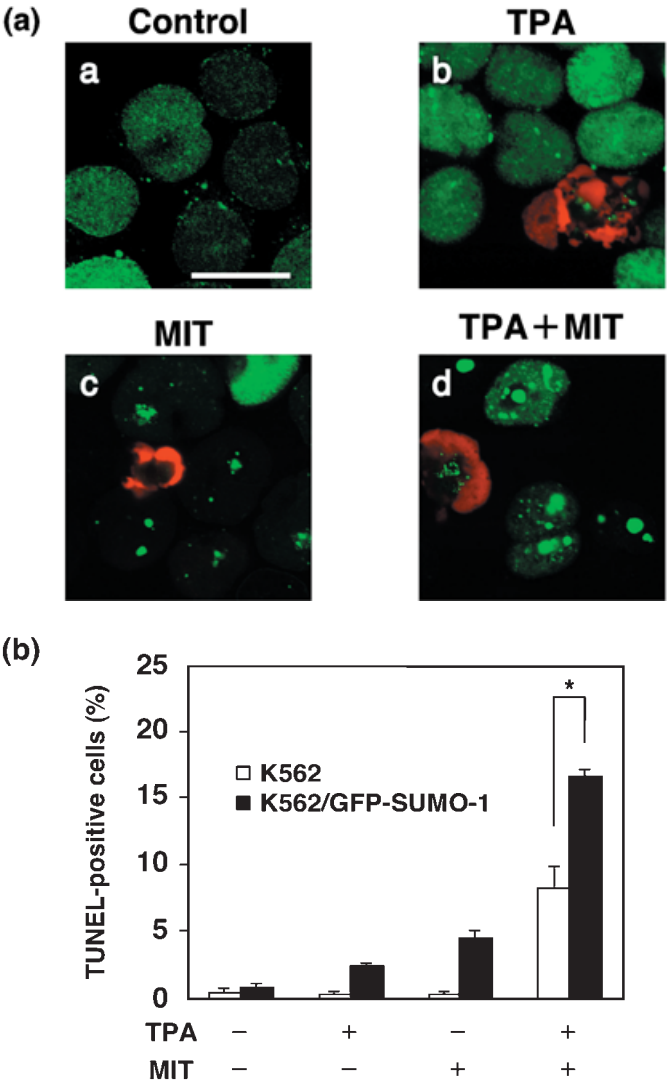

Fig. 1. Relationship between the characteristics of expression of GFP-SUMO-1 and the sensitivity to apoptosis in the treatment of mitoxantrone (MIT) with or without 12-O-tetradecanoylphorbol-13-acetate (TPA). (a) panels a–d, K562/GFP-SUMO-1 cells were treated with or without TPA (2 nM), MIT (0.5 μg/mL), and TPA plus MIT for 24 h, followed by TdT-mediated dUTP nick end labeling (TUNEL) staining. Data were analyzed by laser scanning confocal microscopy. Green and red signals indicate GFP-SUMO-1 and TUNEL-positive cells, respectively. Bar, 20 μm. (b) Wild-type K562 cells and K562/GFP-SUMO-1 cells were treated as described in (a). TUNEL-positive wild-type K562 cells (white bar) and K562/GFP-SUMO-1 cells (black bar) were counted on three different fields counting 200 cells per field. Means ± SD from triplicate determination are shown. *$P < 0.01$.

of treatment with TPA plus MIT, the percentage of TUNEL-positive cells was significantly increased to 16.6 ± 0.5%. Surprisingly, overexpression of GFP-SUMO-1 made the percentage of TUNEL-positive cells increased as compared with wild-type K562 cells. These results suggest that overexpression of SUMO-1 makes the cells more sensitive to induction of apoptosis by MIT, and that the number of apoptotic cells were increased more when the cells form large GFP-SUMO-1 foci.

Blockage of the formation of SUMO-1 foci by siRNA decreases apoptosis. To investigate the direct relationship between the formation of SUMO-1 foci and apoptosis induced by an antitumor reagent, we suppressed the expression of SUMO-1 using siRNA against SUMO-1 and analyzed the effect of siRNA

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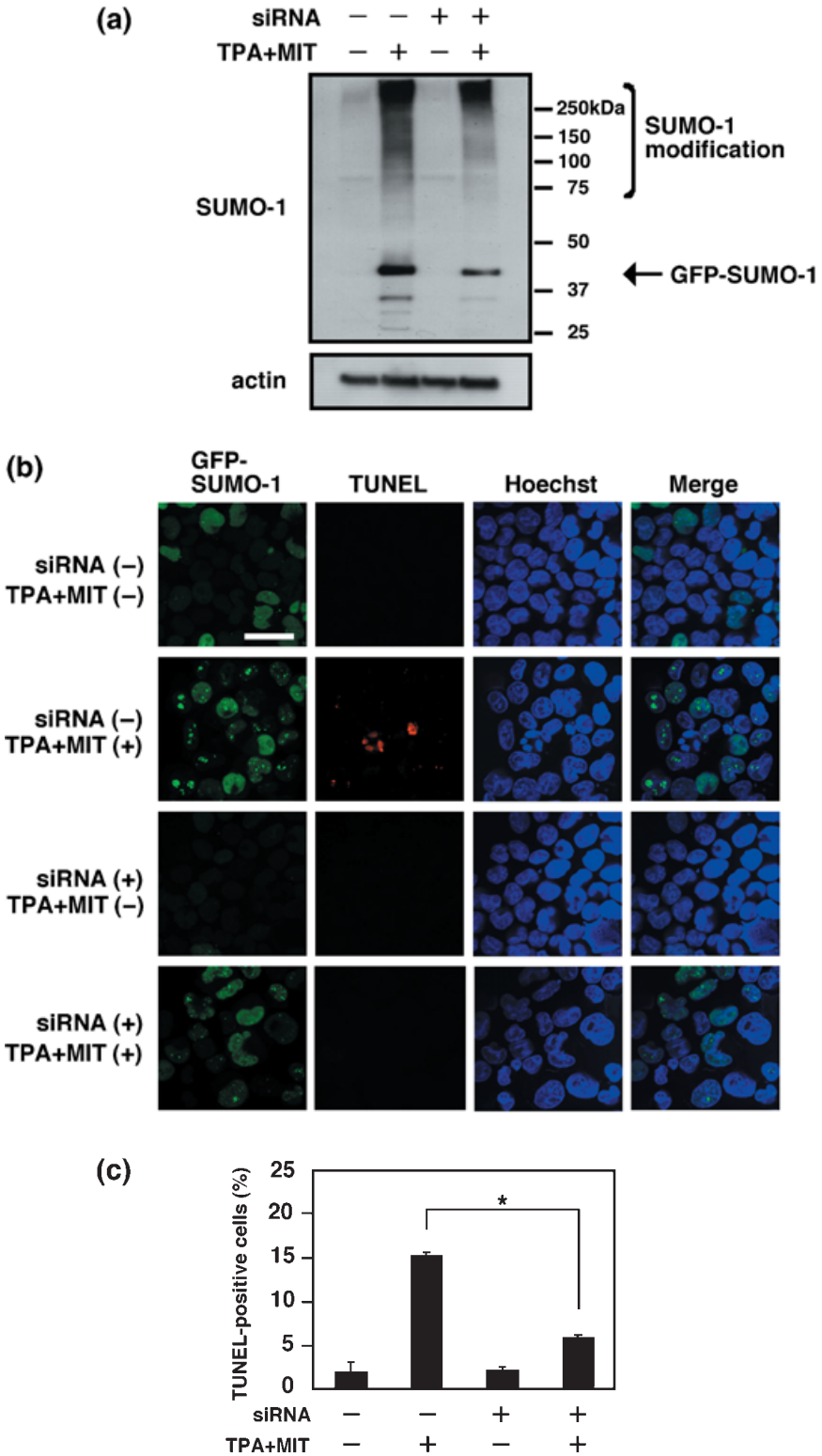

Fig. 2. Blockage of the formation of small ubiquitin-related modifier (SUMO)-1 foci by siRNA decreases apoptosis. (a) The transfection of siRNA for SUMO-1 to K562/GFP-SUMO-1 cells for 48 h, the cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (2 nM) plus mitoxantrone (MIT) (0.5 μg/mL) for 24 h, and lysed with sample buffer. Western blot analysis was performed with anti-SUMO-1 and anti-actin antibodies. (b) The cells were treated as described in (a) followed by TdT-mediated dUTP nick end labeling (TUNEL) staining. Data were analyzed by laser scanning confocal microscopy. Green, red, and blue signals indicate GFP-SUMO-1, TUNEL, and Hoechst 33258, respectively. Bar, 50 μm. (c) TUNEL-positive K562/GFP-SUMO-1 cells were counted on three different fields counting 200 cells per field. Means ± SD from triplicate determination are shown. *$P < 0.01$.

on apoptosis induced by treatment with TPA plus MIT. After siRNA was transfected into K562/GFP-SUMO-1 cells for 48 h, the cells were treated with or without TPA (2 nM) plus MIT (0.5 μg/mL) for 24 h (Fig. 2). On Western blot analysis, in the case of prior treatment of reagents, the expression of GFP-SUMO-1 was weakly positive. However, the expression of GFP-SUMO-1 and the level of sumoylation were increased by treatment with TPA plus MIT. The siRNA partially suppressed the expression of GFP-SUMO-1 and the level of sumoylation increased by treatment with TPA plus MIT (Fig. 2a). When K562/GFP-SUMO-1 cells without transfection of siRNA were treated with TPA plus MIT, the cells formed large GFP-SUMO-1 foci (Fig. 2b, second row). On the other hand, when K562/GFP-SUMO-1 cells with transfection of siRNA were treated with TPA plus

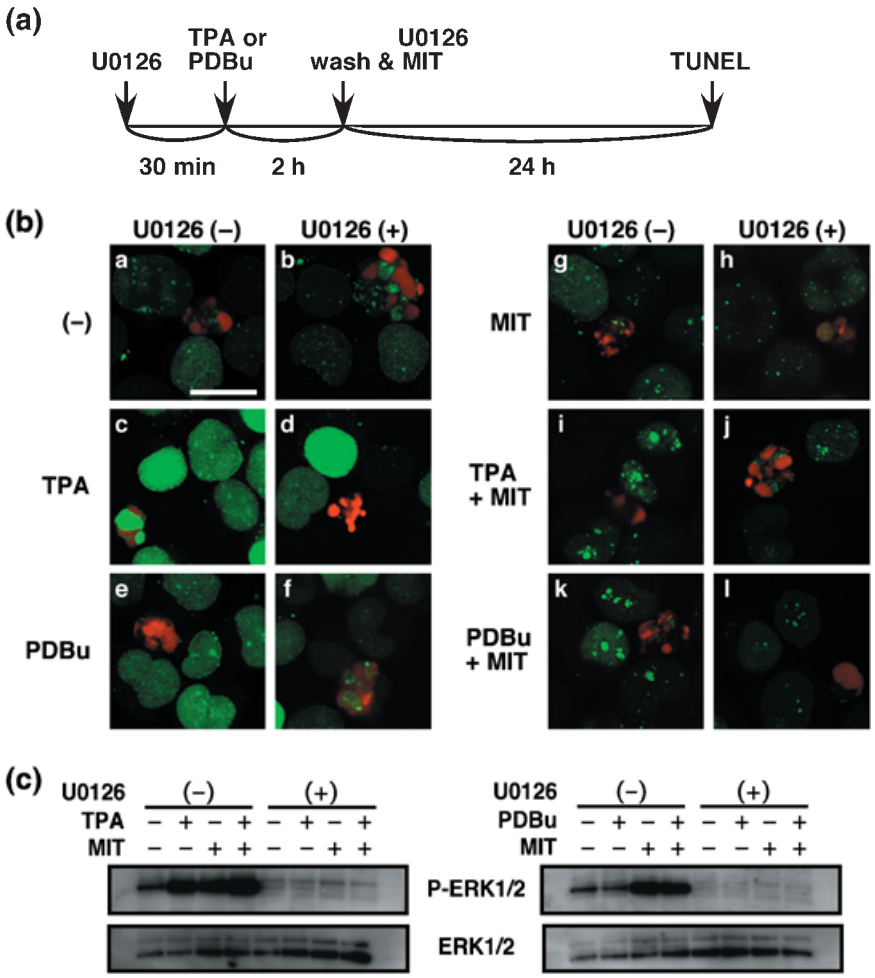

Fig. 3. Inhibition of GFP-SUMO-1 foci formation by U0126. (a) Schedules for treating K562/GFP-SUMO-1 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) (2 nM), phorbol 12, 13-dibutyrate (PDBu) (2 nM), mitoxantrone (MIT) (0.5 μg/mL), and U0126 (10 μM). (b) Panels a-l, the cells were treated as described in (a), followed by TdT-mediated dUTP nick end labeling (TUNEL) staining, and analyzed by laser scanning confocal microscopy. Green and red signals indicate GFP-SUMO-1 and TUNEL-positive cells, respectively. Bar, 20 μm. (c) The cells were treated as described in (a) and lysed with sample buffer. Western blot analysis was performed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies.

MIT, the cells partially suppressed the formation of large GFP-SUMO-1 foci (Fig. 2b, bottom row). The percentage of TUNEL-positive cells with transfection of siRNA was significantly decreased from 15.2 ± 0.4% to 5.9 ± 0.3% ($P < 0.001$) (Fig. 2c). This indicates that siRNA against SUMO-1 could suppress the formation of large GFP-SUMO-1 foci on K562/GFP-SUMO-1 cells, and that it could inhibit apoptosis induced by treatment with TPA plus MIT. These suggest that the formation of large SUMO-1 foci is related to the apoptosis induced by antitumor reagent.

MEK inhibitor U0126 inhibited the formation of large GFP-SUMO-1 foci. Which treatment of TPA or MIT is required for the formation of large GFP-SUMO-1 foci? To answer this question, we examined schedules for treatments with TPA and MIT. Since TPA adheres to the cell surface and is difficult to wash out, PDBu was also used instead of TPA, which is more soluble than TPA in growth medium and was less likely to be retained by cells after wash-out. As a result, pretreatment of TPA or PDBu followed by MIT induced the formation of large GFP-SUMO-1 foci (data not shown). On the other hand, in the case of pretreatment of MIT followed by TPA or PDBu, the formation of only small GFP-SUMO-1 foci was induced (data not shown). These results suggest that pretreatment of TPA or PDBu plays an important role in the promotion of large GFP-SUMO-1 foci formation.

Since TPA and PDBu activate MEK-ERK pathway,[17] we examined the effects of MEK1/2 inhibitor U0126 on the formation of large GFP-SUMO-1 foci by TPA or PDBu and MIT. K562/GFP-SUMO-1 cells were treated with U0126 (10 μM) for 30 min prior to addition of TPA (2 nM) or PDBu (2 nM) and MIT (0.5 μg/mL) as shown in Fig. 3a. In the presence of U0126, the formation of large GFP-SUMO-1 foci by TPA plus MIT or PDBu plus MIT was partially inhibited in size (Fig. 3b, panels i, j, k, and l). When the cells were treated with U0126 and MIT, the GFP-SUMO-1 foci also became smaller as compared with MIT alone (Fig. 3b, panels g and h). The accumulation of GFP-SUMO-1 into the nucleus by TPA or PDBu was suppressed by U0126 (Fig. 3b, panels c, d, e, and f). Furthermore, phosphorylation of ERK1/2 was suppressed clearly by U0126 on Western blot analysis (Fig. 3c). These results suggest that activation of ERK1/2 is required for not only the formation of large GFP-SUMO-1 foci but also the accumulation of GFP-SUMO-1 into the nucleus.

Blockage of ERK1/2 nuclear export by LMB inhibited the formation of large GFP-SUMO-1 foci. ERK can translocate into the nucleus after phosphorylation and activation in the cytoplasm.[19] LMB binds directly to chromosome region maintenance 1 (CRM1), a receptor for the nuclear export signal (NES), and CRM1 is essential for NES-dependent nuclear export of proteins.[20] The export of ERK1/2 from nucleus to cytosol is inhibited by LMB.[21] To examine the effect of LMB on the formation of large GFP-SUMO-1 foci, the K562/GFP-SUMO-1 cells were treated with TPA, PDBu and MIT in the presence or absence of LMB. The optimal concentration and exposure time of LMB were 10 nM and 8 h, respectively. Since the large GFP-SUMO-1 foci, which are formed by MIT even after the removal of TPA or PDBu, were detectable for 14 h under fluorescent microscopy (data not shown), the effect of LMB on the formation of large

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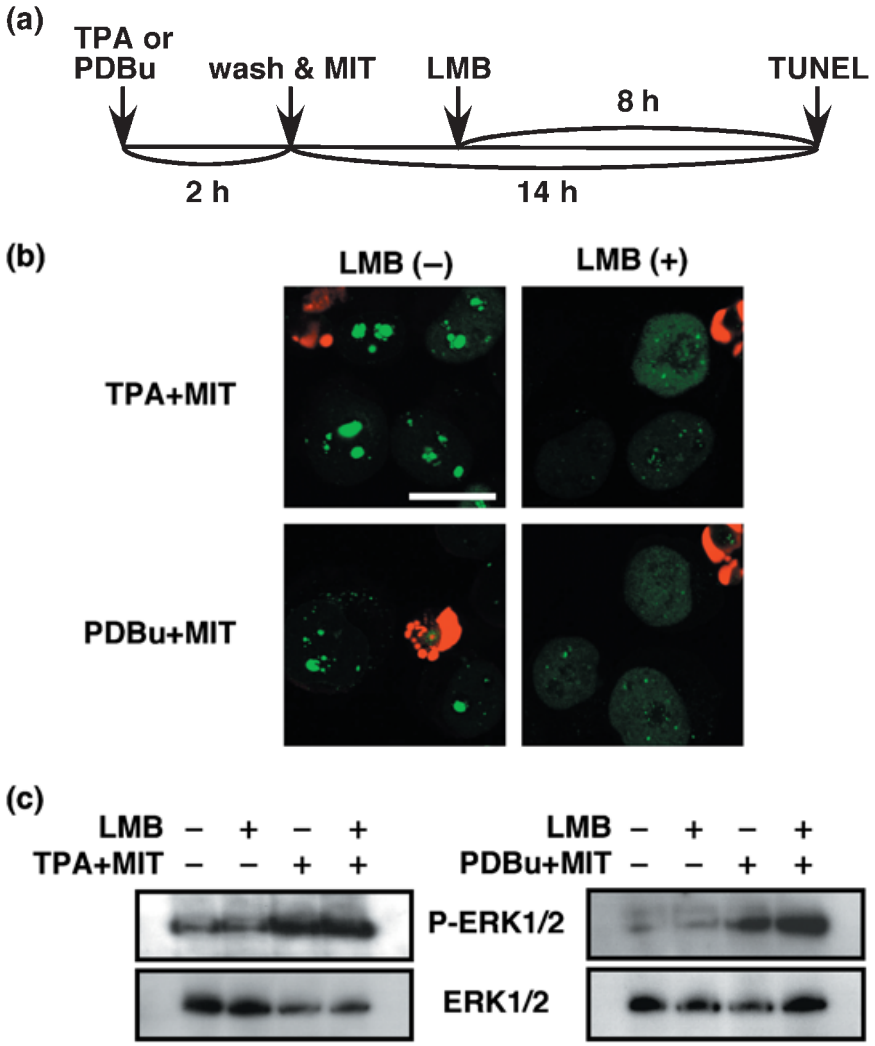

Fig. 4. Inhibition of GFP-SUMO-1 foci formation by leptomycin B (LMB). (a) Schedules for treating K562/GFP-SUMO-1 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) (2 nM), phorbol 12, 13-dibutyrate (PDBu) (2 nm), mitoxantrone (MIT) (0.5 μg/mL), and LMB (10 nM). (b) The cells were treated as described in (a). Data were analyzed by laser scanning confocal microscopy. Green and red signals indicate GFP-SUMO-1 and TdT-mediated dUTP nick end labeling (TUNEL)-positive cells, respectively. Bar, 20 μm. (c) The cells were treated as described in (a) and lyzed with sample buffer. Western blot analysis was performed with antiphospho-ERK1/2 and anti-ERK1/2 antibodies.

GFP-SUMO-1 foci was investigated under these conditions. As shown in Figure 4a, K562/GFP-SUMO-1 cells were treated with or without TPA (2 nM) or PDBu (2 nM) and MIT (0.5 μg/mL) in the presence or absence of LMB (10 nM). In the presence of LMB, the formation of large GFP-SUMO-1 foci by treatment with TPA plus MIT or PDBu plus MIT was inhibited in size (Fig. 4b). Moreover, when the cells were treated with TPA plus MIT or PDBu plus MIT in the presence of LMB, the phosphorylation of ERK1/2 was maintained on Western blot analysis (Fig. 4c). These results suggest that it is necessary for the formation of large GFP-SUMO-1 foci to translocate ERK1/2 between the nucleus and cytoplasm.

Dominant-negative mutant of MEK1 suppressed formation of large SUMO-1 foci. ERK is phosphorylated and activated by an upstream activator kinase, MEK, in response to mitogenic growth factors.[22] To determine if MEK-ERK pathway may participate in the formation of endogenous large SUMO-1 foci, wild-type K562 cells were stably introduced to MEK1 mutant genes, whose lysine 97 was replaced with alanine (K97A), to provide an inactive enzyme. Lysine 97 corresponds with critical lysine found in the ATP-binding site of protein kinases.[23] The mock and the K97A mutant cells were treated with or without TPA (2 nM), MIT (0.5 μg/mL), or TPA plus MIT for 48 h followed by immunofluorescence staining for endogenous SUMO-1. The mock cells formed larger SUMO-1 foci by treatment with TPA plus MIT than with MIT alone (Fig. 5a, panels c and d), whereas the K97A mutant cells partially suppressed the formation of SUMO-1 foci by treatment with MIT alone and TPA plus MIT (Fig. 5a, panels g and h). With TPA treatment, the endogenous SUMO-1 accumulated into the nucleus in the mock cells, whereas the K97A mutant cells suppressed the accumulation of endogenous SUMO-1 into the nucleus (Fig. 5a, panels b and f). In the mock cells, ERK1/2 increased the phosphorylation levels by treatment with TPA alone, MIT alone, and TPA plus MIT for 6 h on Western blot analysis (Fig. 5b). On the other hand, in the K97A mutant cells, the phosphorylation levels of ERK1/2 were suppressed even with treatments of TPA alone, MIT alone, and TPA plus MIT on Western blot analysis (Fig. 5b). To investigate the relationship between the MEK-ERK pathway and apoptosis, the mock cells and the K97A mutant cells were treated with TPA plus MIT for 0 h, 24 h, 48 h, or 72 h followed by TUNEL staining and TUNEL-positive cells were counted (Fig. 5c). When the mock cells were treated with TPA plus MIT, the apoptosis was increased time-dependently and the percentage of TUNEL-positive cells was 25.6 ± 0.8% at 72 h. On the other hand, when the K97A mutant cells were treated for 72 h, the percentage of TUNEL-positive cells was significantly decreased to 7.5 ± 0.5%. These results indicate that activation of ERK1/2 is required for induction of the endogenous large SUMO-1 foci as well as exogenous large GFP-SUMO-1 foci and MEK-ERK pathway activated by TPA plus MIT may participate in apoptosis cascade.

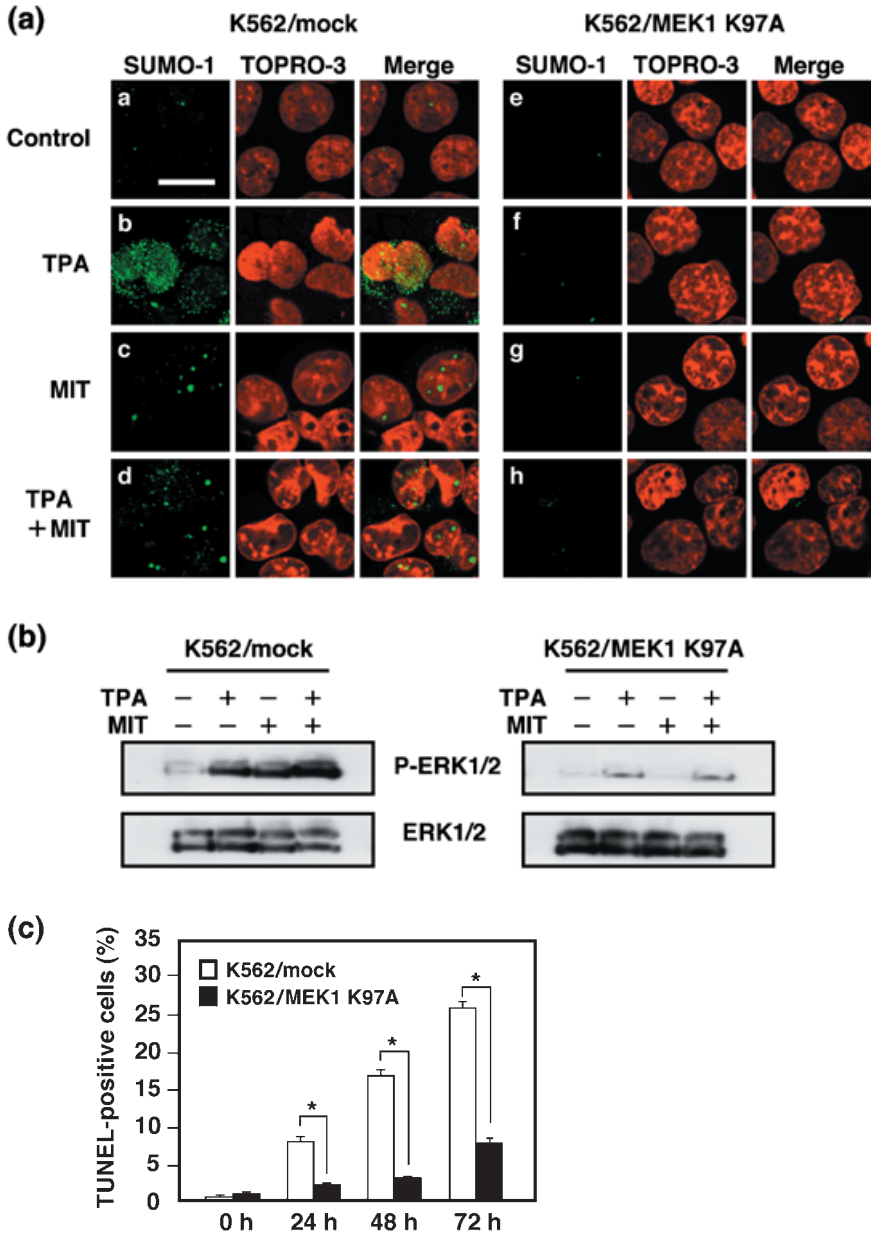

Fig. 5. Inhibition of endogenous small ubiquitin-related modifier (SUMO) foci formation in MEK1 mutant K97A. (a) Panels a-h, K562 cells were transfected with mock (K562/mock) and MEK1 mutant K97A (K562/MEK1 K97A), and treated with or without 12-O-tetradecanoylphorbol-13-acetate (TPA) (2 nM), mitoxantrone (MIT) (0.5 μg/mL), and TPA plus MIT for 48 h. The cells were fixed and immunostained with anti-SUMO-1 antibody followed by Alexa Fluor 488 conjugated antisecondary antibody, and then the nuclei were counter-stained with TOPRO-3. Data were analyzed by laser scanning confocal microscopy. Green and red signals indicate endogenous SUMO-1 and TOPRO-3, respectively. Bar, 20 μm. (b) The cells were treated with TPA (2 nM), MIT (0.5 μg/mL), and TPA plus MIT for 6 h and lysed with sample buffer. Western blot analysis was performed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. (c) The cells were treated with TPA plus MIT for 0 h, 24 h, 48 h, or 72 h followed by TdT-mediated dUTP nick end labeling (TUNEL) staining. TUNEL-positive K562/mock cells (white bar) and K562/MEK1 K97A cells (black bar) were counted on three different fields counting 200 cells per field. Means ± SD from triplicate determination are shown. *$P < 0.01$.

Discussion

In this study, we have performed a characterization of GFP-SUMO-1 subcellular distribution by treatment with or without an antitumor reagent MIT, in the presence or absence of a tumor promoter TPA. The intracellular GFP-SUMO-1 showed different types of subcellular distribution. No researcher has reported these differences in the patterns of SUMO-1 distribution. Moreover, we have also demonstrated that the treatment with TPA plus MIT induced larger SUMO-1 foci than MIT alone.

The patterns of subcellular distribution of GFP-SUMO-1 were schematized as shown in Fig. 6. In the case before treatment of reagents, there are some small GFP-SUMO-1 particles (black circles) in the nucleus (Fig. 6a). These small SUMO-1 particles are modified to the targeted proteins or unmodified. In the case of TPA alone, the number of GFP-SUMO-1 particles increases dramatically, and GFP-SUMO-1 particles are expressed homogeneously in the nucleus (Fig. 6b). On the other hand, in the case of MIT alone, more than two GFP-SUMO-1 particles form some small foci in the nucleus (Fig. 6c). It is possible that the GFP-SUMO-1 particles accumulate and form small foci in a nucleus by MIT. Moreover, after treatment of TPA plus MIT, GFP-SUMO-1 foci become larger than treatment of MIT alone, with some GFP-SUMO-1 particles and small foci in the nucleus at the same time (Fig. 6d).

The expression of GFP-SUMO-1 was increased by treatment with TPA plus MIT (Fig. 2a). The expression vector of GFP has a CMV promoter which enhances the transcriptional activity of genes by treatment with TPA.[24] Because the expression of mRNA and protein of GFP-SUMO-1 were increased by treatment with TPA (data not shown), it is possible that TPA enhanced the transcriptional activity of CMV promoter and the expression of GFP-SUMO-1.

How are the SUMO-1 foci formed? To answer this question, we demonstrated that the phosphorylation of ERK and translocation of ERK from the nucleus to the cytoplasm are important for the formation of large SUMO-1 foci, based on studies of the MEK inhibitor U0126, the nuclear export inhibitor LMB, and

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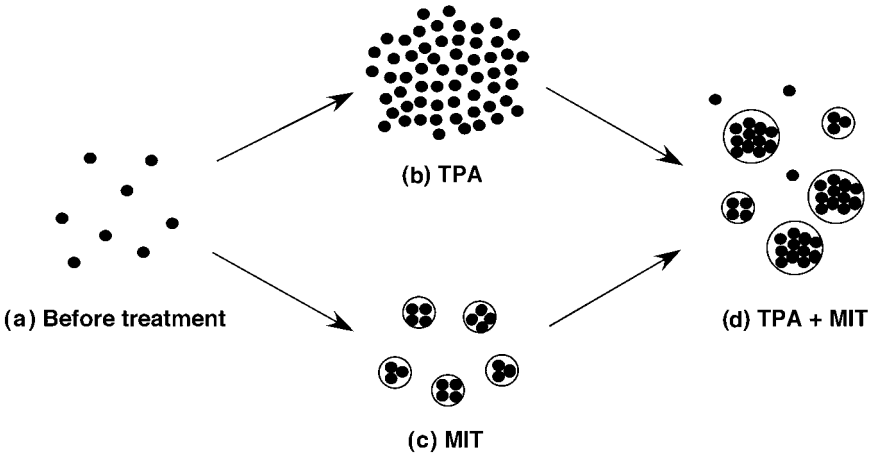

Fig. 6. The transition of GFP-SUMO-1 expression by 12-O-tetradecanoylphorbol-13-acetate (TPA) and mitoxantrone (MIT). (a) Before treatment: there are some GFP-SUMO-1 particles in the nucleus (black circles). (b) TPA treatment: the expression of GFP-SUMO-1 particles is increased dramatically and homogeneously in the nucleus. (c) MIT treatment: more than two GFP-SUMO-1 particles accumulate and form small foci in the nucleus. (d) TPA plus MIT treatment: the GFP-SUMO-1 focus becomes larger than with MIT alone. Furthermore, some particles and small foci (MIT type) are also mixed in the nucleus.

MEK1-dominant negative cells. Since phorbol esters, such as TPA and PDBu, alter gene expression,[25,26] affect protein synthesis,[27] inhibit cellular proliferation, and stimulate differentiation,[28] the formation of large SUMO-1 foci by treatment with TPA plus MIT leads some substrates, which accumulate into the nucleus or whose translation is promoted by TPA-activated ERK pathway, to modification by sumoylation and accumulation into foci by TPA plus MIT. Moreover, since MIT also activates the ERK pathway (Fig. 3c), it is possible that the synergistic effect of TPA and MIT induces the activation of ERK pathway more strongly, and the SUMO-1 foci become larger than MIT alone.

Although we demonstrated that the ERK pathway is related to the formation of SUMO-1 foci by MIT, it is still unclear whether ERK is a direct substrate for sumoylation and which ERK itself or ERK-related proteins participate in the formation of SUMO-1 foci. It has been reported that a dynamic interplay exists between sumoylation and ERK pathway. SUMO-1 modified the EST domain transcriptional factor Elk-1, which is a direct target of ERK pathway, and the SUMO-1 modification is reversible after ERK-mediated Elk-1 activation.[29] Sumoylation is a regulator of Elk-1 function that acts by controlling Elk-1 nuclear presence.[30] In our study, the formation of SUMO-1 foci was promoted by ERK pathway activation.

We also demonstrated that overexpression of GFP-SUMO-1 increases the sensitivity of the cells to antitumor reagent and that the formation of SUMO-1 foci is implicated in the induction of apoptosis by treatment with antitumor reagent. It is well documented that the ERK cascade is stimulated by growth-related signals and is related to cell proliferation or differentiation, whereas the c-Jun – N-terminal kinase (JNK) and p38 MAPK cascades are activated by various stress stimuli and is related to stress response or apoptosis. In contrast, it has been reported that CDDP-induced apoptosis activates ERK pathway followed by caspase-3 activation.[31,32] From our data, the activation of ERK pathway induced apoptosis and it is possible that ERK pathway activated by TPA and MIT may participate in an apoptosis cascade (Fig. 5c).

It is not entirely clear which substrates exist in SUMO-1 foci. SUMO-1 modification of homeodomain-interacting protein kinase 2 (HIPK2),[33] aryl hydrocarbon receptor nuclear transporter (ARNT),[34] PML,[5] or Sp100,[9] is associated with their localization to nuclear dots. PML and Sp100 showed the colocalization with GFP-SUMO-1 foci in the case of MIT or TPA plus MIT treatment (data not shown). Topoisomerases are also substrates of sumoylation. Topoisomerase inhibitors strongly stimulate sumoylation and ubiquitination of topoisomerases, and they form a cleavable complex followed by degradation in 26S proteasome.[11–14] Since MIT is a topoisomerase II inhibitor, it is possible that SUMO-1 foci formed by MIT or TPA plus MIT are also the cleavable complexes of topoisomerase II.

In conclusion, this study demonstrated that the formation of large SUMO-1 foci is related to apoptosis induced by treatment with MIT and is required for activation of ERK pathway or translocation of ERK between the nucleus and the cytoplasm. Thus, further investigation of the relationship between the intracellular distribution of SUMO-1 and the sensitivity to apoptosis induced by several antitumor reagents, would apply for the diagnosis of cancer chemotherapy.

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