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Functional Involvement of mSos in Interleukin-3 and Thrombin Stimulation of the Ras, Mitogen-Activated Protein Kinase Pathway in BaF3 Murine Hematopoietic Cells

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Stimulation of the interleukin (IL)-3 receptor provokes rapid activation of the Ras pathway in various hematopoietic cell lines. Also, a wide range of G-protein-coupled receptors induce Ras activation following ligand stimulation. In this report, we investigate the mechanism underlying Ras activation upon stimulation of these two types of receptors in hematopoietic cells. Thrombin, a G-protein-coupled receptor ligand, was found to stimulate extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) in IL-3-dependent BaF3 cells, suggesting a significant function of thrombin receptor-mediated signaling. We show that the Ras-guanine nucleotide exchange factor mSos is indispensable for activation of the Ras pathway in IL-3- or thrombin-stimulated BaF3 cells. The activation of Ras in response to IL-3 as defined by accumulation of the GTP-bound form was impaired by conditional overexpression of a dominant-negative mutant of mSos (⊿mSos1). Furthermore, following induction of ⊿mSos1, IL-3 enhancement of the kinase activities of c-Raf-1, ERK2, and JNK1 downstream of Ras was almost completely blocked. Similarly, thrombin-induced Ras-dependent ERK2 activation was diminished by ⊿mSos1. However, the tyrosine phosphorylation pattern of cellular substrates upon thrombin stimulation was entirely different from the pattern of IL-3-induced tyrosine phosphorylation. Collectively, these results provide evidence that mSos plays a crucial role in both IL-3 and thrombin activation of the Ras pathway in hematopoietic cells, although molecules (including tyrosine kinases) mediating the signal to mSos are likely to be different between the two types of receptors.

Key words: interleukin-3, mSos, Ras, thrombin.

Ras family GTP-binding proteins regulate cell growth and differentiation in a wide variety of mammalian cells (*1, 2*). In cells of the hematopoietic lineage, stimulation with various cytokines, including interleukin (IL)-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, and erythropoietin, elicits Ras activation as evidenced by accumulation of the GTP-bound form, suggesting a significant role of Ras in cytokine receptor-mediated signal transduction (*3-5*). Despite experiments intended to define the physiological function of Ras in cytokine-promoted signal transduction, however, this remains controversial (*6-9*). Diverse trimeric G-protein-coupled receptors also stimulate the Ras pathway in various cell lines (*10-14*). Further, overexpression of G-protein $\beta\gamma$ subunits in COS cells leads to Ras activation, suggesting a role of $\beta\gamma$ subunits in receptor-mediated signal transduction (*15-17*).

In signaling pathways mediated by tyrosine kinase-type receptors, such as platelet-derived growth factor, epidermal growth factor, and insulin receptors, intermediate signaling components between the receptor and Ras are well characterized (*18, 19*). Of these, mSos has been implicated as a key element. Two ubiquitously expressed mSos proteins, mSos1 and mSos2, are identified in mammalian cells as homologues of the *Drosophila melanogaster* SOS protein (*20*). They directly interact with Ras leading to enhanced exchange of the Ras-bound guanine nucleotide. Ligand stimulation causes tyrosine phosphorylation at specific sites of the receptor, thereby triggering the association of adaptors such as Shc, Grb2, and Crk with the receptor through the specific interaction between a phosphorylated tyrosine and the Src homology (SH) 2 or phosphotyrosine-binding domain. Cytoplasmic mSos, which forms a stable complex with Grb2 through the interaction between the PXXP (where P and X stand for proline and any amino acid, respectively) motif and the SH3 domain, translocates to the plasma membrane along with Grb2, thereby stimulating the GDP/GTP exchange reaction of membrane-associated Ras proteins. Conservation of this signaling cascade among mammalian, fruit fry, and nematode cells was remarkably helpful for identifying the components. In principle, attachment of mSos to the

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Abbreviations: IL, interleukin; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SH, Src homology; JAK, *Janus* kinase; GST, glutathione *S*-transferase; TRP, thrombin receptor agonist peptide; PTX, pertussis toxin; IPTG, isopropyl-1-thio-β-D-galactoside.

plasma membrane is sufficient to facilitate the Ras signaling pathway because targeting to the membrane by the use of a lipid modification or a chemical inducer of dimerization confers a constitutive Ras-stimulating activity to mSos (*21-23*). However, signal transduction mediated by Grb2 and mSos actually seems more complicated. Indeed, by using a transient expression system, it is suggested that pleckstrin homology and Dbl homology domains at the amino-terminal portion of mSos, rather than the carboxy-terminal Grb2 SH3-binding domain, are required for Ras activation (*24, 25*). Furthermore, an important role for the pleckstrin homology domain in signal-dependent membrane targeting of mSos was described (*26*).

Although cytokine receptors, such as IL-2 and IL-3 receptors, exhibit no tyrosine kinase activity, stimulation of several kinds of non-receptor-type tyrosine kinases, including Src family and *Janus* kinase (JAK) family kinases, is crucial for the signal transduction (*27*). Following activation of the tyrosine kinases, tyrosine phosphorylation of Shc and complex formation among the receptor, Shc, Grb2, and mSos occur, which is considered to elicit subsequent Ras activation in a manner similar to tyrosine kinase-type receptor-mediated signaling (*9, 28-35*). However, at present, it remains to be solved whether the Shc-Grb2-mSos pathway is indispensable for cytokine-induced Ras activation. The mechanism governing Ras activation downstream of G-protein-coupled receptors is also incompletely understood. Ras activation mechanisms through certain members of this receptor family, including α_{2A} adrenergic and lysophosphatidic acid receptors, were investigated in COS cells, elucidating that the Shc-Grb2-mSos pathway is responsible for Ras activation (*36*).

In the present study, we evaluate the functional role of mSos in IL-3 or G-protein-coupled receptor-induced activation of the Ras signaling pathway in hematopoietic cells. Thrombin was found to stimulate extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) through its G-protein-coupled receptor in mouse IL-3-dependent hematopoietic BaF3 cells. These results allowed us to compare signaling pathways regulated by two distinct types of receptors, *i.e.*, IL-3 and thrombin receptors, within the same cell line. Using a stable clone that conditionally expresses a dominant-negative mutant of mSos1, termed ⊿mSos1 (*37*), we show that ⊿mSos1 substantially impairs IL-3-induced Ras activation as determined by accumulation of the GTP-bound active form of Ras. As a consequence, the increase in the activities of downstream kinases upon IL-3 treatment is markedly diminished. Thrombin-induced Ras-dependent activation of ERK2 was also sensitive to the inhibitory effect of ⊿mSos1. However, tyrosine phosphorylation of cellular proteins, which is remarkable upon IL-3 stimulation, was observed only weakly and transiently in the case of thrombin signal transduction. Taken together, the association of mSos with Grb2 is likely to be crucial for both IL-3- and thrombin-induced signal transduction leading to Ras activation, although the intermediate molecules, including tyrosine kinases, downstream of their receptors are distinguishable from each other.

MATERIALS AND METHODS

Materials—Full-length cDNAs encoding mouse Sos1 (*20*) and human Grb2 (*38*) were generous gifts of David Bowtell (University of Melbourne, Parkville, Victoria, Australia) and Joseph Schlessinger (New York University Medical Center, NY), respectively. An *Escherichia coli* expression system of His-MAPKK and glutathione *S*-transferase (GST)-MAPK(K57D) was kindly provided by Eisuke Nishida (Kyoto University, Kyoto). His-MAPKK and GST-MAPK(K57D) were purified by use of nickel and glutathione columns, respectively. A plasmid for expression of GST-c-Jun(1-223) in *E. coli* was kindly provided by Michael Karin (University of California San Diego, La Jolla, CA). GST-c-Jun(1-223) was purified using a glutathione column. Purified mouse IL-3 was a generous gift of Satish Menon (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Thrombin receptor agonist peptide (TRP; the amino acid sequence: SFLLRNPN-DKYEPF) was a generous gift of Masahiro Takada and Osamu Ito (Tsukuba Research Laboratories, Eisai, Tsukuba). Antibodies against ERK2 (sc-154), c-Raf-1 (sc-227), Grb2 (sc-255), mSos1/2 (sc-259), JAK2 (sc-278), and JNK1 (sc-474) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (05-321) and anti-Shc (06-203) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Human thrombin (T-6759) and pertussis toxin (PTX) (KK-200) were purchased from Sigma Chemical (St. Louis, MO) and Kaken Pharmaceutical (Tokyo), respectively.

Cell Culture—BaF3 cells were cultured in RPMI 1640 supplemented with fetal calf serum [10% (v/v)], mouse IL-3 (approximately 1 nM). BaF3-N6 (*9*) and BaF3-⊿mSosA6 cells were cultured in RPMI 1640 supplemented with fetal calf serum [10% (v/v)], mouse IL-3 (approximately 1 nM), G418 (1 mg/ml), and hygromycin B (0.8 mg/ml). For starvation, cells were incubated in RPMI 1640 supplemented with bovine serum albumin (1 mg/ml) for 3 h.

Plasmid Construction and Isolation of Stable Transfectants of the BaF3 Cell Line—The cDNA encoding ⊿mSos1 (mSos1 lacking amino acids 577-1073) was constructed according to a previous report (*37*). The fragment encoding ⊿mSos1 was subcloned into the expression vector pEF-LAC (*39*), generating pEF-LAC-⊿mSos1. pEF-LAC-⊿mSos1 (30 μg) and p3′SS of the lactose operator/repressor system (Stratagene, La Jolla, CA) (15 μg) were introduced into BaF3 cells (10^7 cells) by electroporation, and transfectants were selected in the presence of G418 (1 mg/ml) and hygromycin B (0.8 mg/ml) essentially as described previously (*40*).

Immunoprecipitation and Immunoblotting—Cells were dissolved in IP buffer [50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM $MgCl_2$, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 1 μg/ml leupeptin], centrifuged at 15,000 × *g* for 15 min at 4°C, and the supernatant was used as a cell lysate. Protein A-Sepharose CL-4B (Phamacia Biotech) and a specific antibody were mixed gently with the lysate for 2 h at 4°C, and the precipitate was washed five times with IP buffer. The precipitated proteins were then separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was stained with a specific antibody and enhanced chemiluminescence detection reagents (DuPont/NEN).

Analysis of Ras-Bound GDP and GTP—The analysis of Ras-bound GDP and GTP in BaF3-⊿mSosA6 cells was performed as described elsewhere (*41*).

Kinase Assay of c-Raf-1—Cells were lysed in kinase IP buffer [20 mM Tris-HCl (pH 7.5), 0.5% (v/v) Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 3 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride], centrifuged at 15,000×*g* for 15 min at 4°C, and the supernatant was mixed with protein A-Sepharose CL-4B (Phamacia Biotech) and an anti-c-Raf-1 antibody (sc-227, 0.25 μg). The mixture was incubated for 2 h at 4°C with gentle mixing, and the precipitate was washed twice with kinase IP buffer, twice with kinase wash buffer [20 mM Hepes-NaOH (pH 7.6), 0.05% (v/v) Nonidet P-40, 50 mM NaCl, 0.1 mM EDTA], and once with Raf reaction buffer [20 mM Hepes-NaOH (pH 7.0), 5 mM $MgCl_2$, 1.5 mM NaF, 3.75 mM β-glycerophosphate, 0.15 mM Na_3VO_4]. The precipitated proteins were subjected to the kinase assay within Raf reaction buffer (30 μl) containing 23.7 μg/ml His-MAPKK, 172 μg/ml GST-MAPK(K57D), and 0.2 mM [γ-^{32}P]ATP (37 TBq/mol) for 20 min at 30°C. The proteins were separated by SDS-PAGE, and the radioactivity incorporated into GST-MAPK(K57D) was quantitated by an image analyzer (BAS2000, Fuji Film, Tokyo).

Kinase Assay of ERK/MAPK and JNK/SAPK—Cells were lysed in kinase IP buffer, centrifuged at 15,000×*g* for 15 min at 4°C, and the supernatant was mixed with protein A-Sepharose CL-4B (Phamacia Biotech) and an anti-ERK2 (sc-154, 1 μg) or anti-JNK1 (sc-474, 0.25 μg) antibody. The mixture was incubated for 2 h at 4°C with gentle mixing, and the precipitate was washed twice with kinase IP buffer, twice with kinase wash buffer, and once with MAPK reaction buffer [25 mM Hepes-NaOH (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 2 mM dithiothreitol]. The precipitated proteins were subjected to the kinase assay within MAPK reaction buffer (30 μl) containing 0.25 mg/ml myelin basic protein (for ERK/MAPK) or 0.033 mg/ml GST-c-Jun(1-223) (for JNK/SAPK), and 20 μM [γ-^{32}P]ATP (307 TBq/mol) for 20 min at 30°C. The proteins were separated by SDS-PAGE, and the radioactivity incorporated into each substrate was quantitated by use of an image analyzer (BAS2000, Fuji Film).

RESULTS

To clarify mechanisms underlying Ras activation in response to stimulation with cytokine and G-protein-coupled receptors in hematopoietic cells, we examined several G-protein-coupled receptor ligands for their ability to stimulate IL-3-dependent BaF3 cells. Among tested ligands, thrombin was found to induce ERK2 activation in a dose-dependent manner as shown in Fig. 1A. Likewise, TRP, a peptide mimicking the new amino terminus created by cleavage of the thrombin receptor (*42*), stimulated ERK2 activity at concentrations where the thrombin receptor is known to be activated (Fig. 1B). As demonstrated in Fig. 1C, treatment with PTX abolished thrombin-induced ERK2 activation, suggesting that G_i-type G proteins are coupled to the thrombin receptor to stimulate the ERK/MAPK pathway in the BaF3 cell line.

Effects of dominant-negative Ras(S17N) on IL-3- and

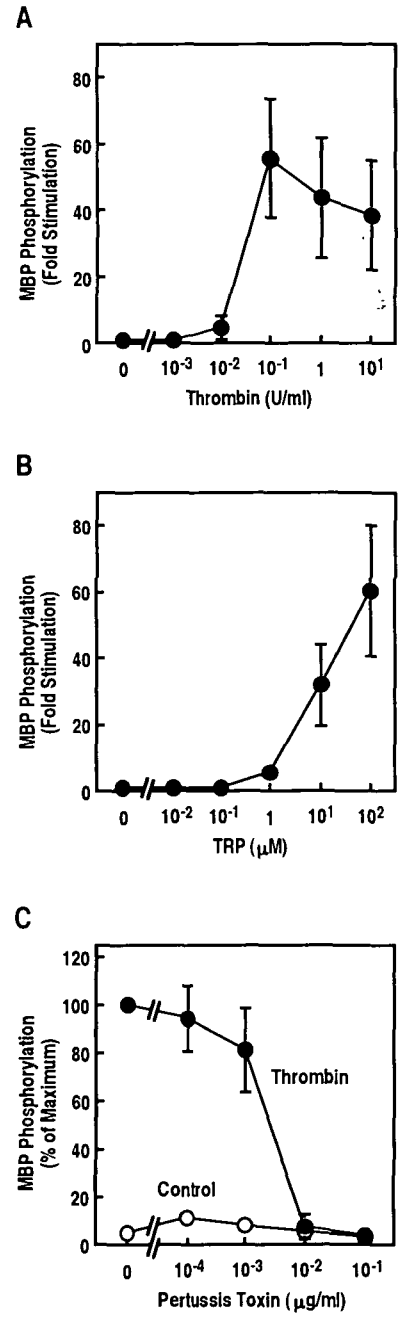


Fig. 1. **Thrombin-induced ERK2 activation in BaF3 cells.** (A) Thrombin-induced activation of ERK2. Following starvation for 3 h, BaF3 cells were stimulated with indicated concentrations of human thrombin for 3 min. Fold stimulation of the activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD (n=3). (B) TRP-induced activation of ERK2. Following starvation for 3 h, BaF3 cells were stimulated with indicated concentrations of the agonist peptide TRP for 3 min. Fold stimulation of the activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD (n=3). (C) Effects of PTX on thrombin-induced ERK2 activation. BaF3 cells were incubated in the culture medium containing indicated concentrations of PTX for 24 h. Following starvation for 3 h, the cells were stimulated with (●) or without (○) human thrombin (1 U/ml) for 3 min. The relative activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD (n=3).

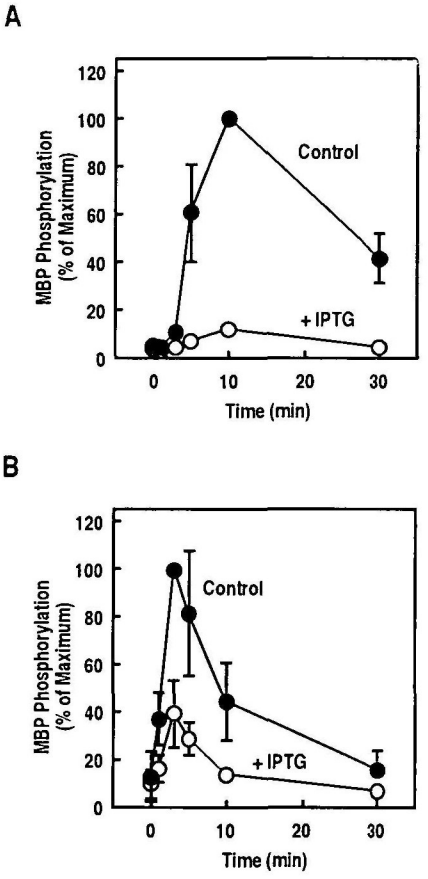


Fig. 2. **Effects of Ras(S17N) on IL-3- or thrombin-induced ERK2 activation.** (A) Inhibition of IL-3-induced ERK2 activation by Ras(S17N). BaF3-N6 cells were incubated in the culture medium with (○) or without (●) IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with mouse IL-3 (25 ng/ml) for indicated periods. The relative activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD ($n=3$). (B) Inhibition of thrombin-induced ERK2 activation by Ras(S17N). BaF3-N6 cells were incubated in the culture medium with (○) or without (●) IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with human thrombin (1 U/ml) for indicated periods. The relative activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD ($n=3$).

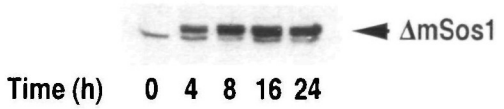
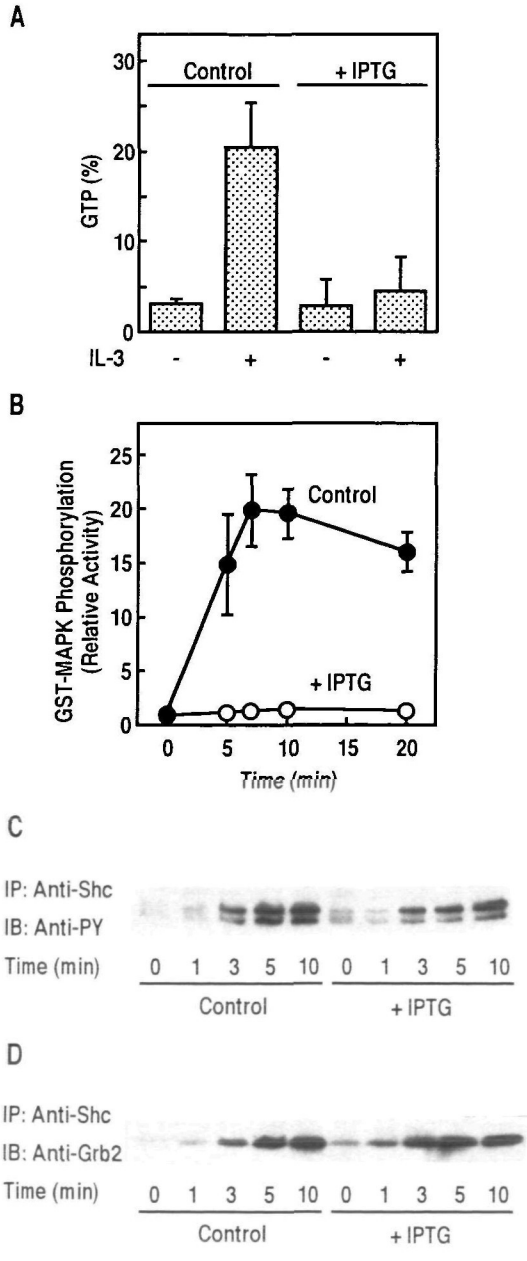


Fig. 3. **IPTG induction of ⊿mSos1 in BaF3-⊿mSosA6 cells.** BaF3-⊿mSosA6 cells were incubated in the culture medium containing IPTG (5 mM) for indicated periods. The ⊿mSos1 protein stained with an anti-mSos1/2 antibody (sc-259) is shown.

thrombin-induced ERK2 activation are shown in Fig. 2. The BaF3-N6 cell is a BaF3-derived transfectant that conditionally expresses Ras(S17N) upon isopropyl-1-thio-β-D-galactoside (IPTG) treatment (*9*). IL-3-dependent ERK2 activation was entirely dependent on Ras as evidenced by the inhibitory effect of dominant-negative Ras(S17N) (Fig. 2A), as previously described (*9, 43*). To determine whether thrombin-induced activation of ERK2 is mediated by Ras, the effect of Ras(S17N) was examined. As is the case for the parental BaF3 cell line, thrombin potently stimulated ERK2 in BaF3-N6 cells (Fig. 2B). However, in contrast with IL-3 stimulation, where maximal ERK2 activation was achieved 10 min after the addition of IL-3, thrombin induction peaked at 3 min, rapidly decreasing thereafter. Moreover, while IL-3-induced ERK2 activation was completely abrogated upon induction of Ras(S17N), Ras(S17N) prevented thrombin-dependent ERK2 activation up to approximately 40% of the maximal level. Thus, it is likely that both Ras-dependent and independent signaling pathways may regulate ERK2 activity in response to thrombin.

To manifest the role of mSos in IL-3- and thrombin-stimulated Ras activation in BaF3 cells, we isolated stable transfectants that express dominant-negative mSos in an inducible manner. The deletion mutant ⊿mSos1, which lacks the Cdc25-related catalytic domain for stimulation of Ras-GDP/GTP exchange, but is capable of binding to an



adaptor such as Grb2, has been demonstrated to act in a dominant-negative manner (*37*). We isolated BaF3-derived stable transfectants that conditionally express ⊿mSos1 upon IPTG treatment. One of the isolated clones, designated BaF3-⊿mSosA6, was further characterized in the present study. The time course of accumulation of the ⊿mSos1 protein following IPTG induction of the EF-LAC promoter in BaF3-⊿mSosA6 cells is illustrated in Fig. 3. Accumulation of ⊿mSos1 was significant after 4 h of treatment with IPTG, reached a maximum at 16 h, and was sustained for at least an additional 8 h. The maximal expression level of ⊿mSos1 estimated by immunoblotting was approximately 27-fold higher than that of endogenous mSos. The lower bands seem unrelated to ⊿mSos1 since they also appear in the blot of untransfected cells. The mSos-mediated pathway is blocked provided that catalytically inactive ⊿mSos1 sufficiently prevents the interaction between adaptors such as Grb2 and endogenous mSos by sequestering the former. We examined association between ⊿mSos1 and Grb2 with the use of a transient expression assay in human embryonic kidney 293 cells. Both full-length mSos1 and ⊿mSos1, when overproduced, were co-immunoprecipitated with Grb2, and the binding of Grb2 to endogenous mSos1 was efficiently blocked (data not shown) as previously described in CHO stable transfectants (*37*). The results indicate that overexpression of ⊿mSos1 prevents the interaction of endogenous mSos with Grb2, thus blocking the signal transduction.

First, we examined the effect of ⊿mSos1 on the Ras-bound GDP/GTP state, which represents Ras activity. As shown in Fig. 4A, 10 min of stimulation with IL-3 facilitated accumulation of the GTP-bound form of Ras. When the cells were treated with IPTG for inducing ⊿mSos1 prior to IL-3 stimulation, the IL-3-stimulated increase in the Ras•GTP level was impaired almost completely, indicating that the mSos1-mediated signaling pathway is crucial for IL-3-induced Ras activation. Consistent with this, IL-3 induction of the kinase activity of c-Raf-1, which directly binds to Ras•GTP and is subsequently activated in a Ras-dependent manner, was completely blocked by ⊿mSos1 (Fig. 4B). In marked contrast, IL-3-induced tyrosine phosphorylation of

Fig. 4. **Inhibition of IL-3-induced activation of Ras and c-Raf-1, but not Shc-Grb2 signaling, by ⊿mSos1.** (A) Inhibition of Ras activation. BaF3-⊿mSosA6 cells were incubated in the culture medium with or without IPTG (5 mM) for 24 h. Following starvation and concomitant labeling with [^{32}P]orthophosphate for 3 h, the cells were stimulated with mouse IL-3 (25 ng/ml) for 10 min or left unstimulated. The ratio of Ras•GTP *versus* Ras•GDP+Ras•GTP is shown as mean±SD ($n=3$). (B) Inhibition of c-Raf-1 activation. BaF3-⊿mSosA6 cells were incubated in the culture medium with (○) or without (●) IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with mouse IL-3 (25 ng/ml) for indicated periods. The relative activity of c-Raf-1 immunoprecipitated with a specific antibody (sc-227) is shown as mean±SD ($n=3$). (C) Tyrosine phosphorylation of Shc. BaF3-⊿mSosA6 cells were incubated in the culture medium with or without IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with mouse IL-3 (25 ng/ml) for indicated periods. Tyrosine phosphorylation of Shc was examined by immunoprecipitation with an anti-Shc antibody (06-203) and subsequent immunoblotting with an anti-phosphotyrosine antibody (05-321). Bands corresponding to p52^{Shc} and p46^{Shc} are shown. (D) Association of Shc with Grb2. The same blot as in (B) was probed with an anti-Grb2 antibody (sc-255). Bands corresponding to Grb2 are shown.

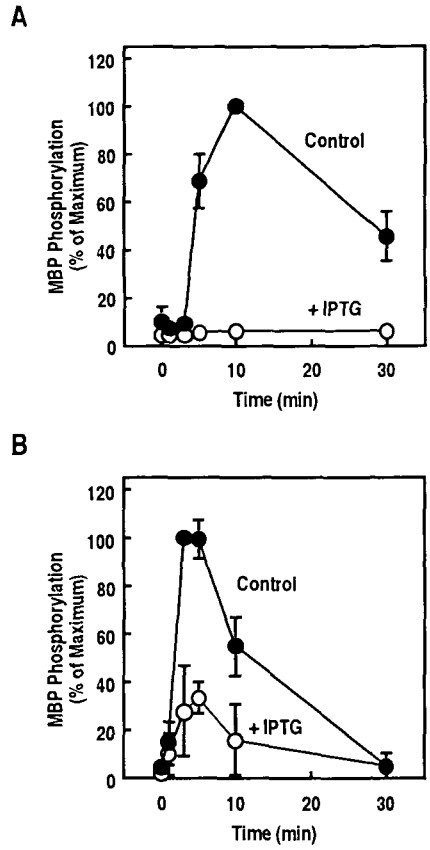


Fig. 5. **Effects of ⊿mSos1 on IL-3- or thrombin-induced ERK2 activation.** (A) Inhibition of IL-3-induced ERK2 activation by ⊿mSos1. BaF3-⊿mSosA6 cells were incubated in the culture medium with (○) or without (●) IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with mouse IL-3 (25 ng/ml) for indicated periods. The relative activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD ($n=3$). (B) Inhibition of thrombin-induced ERK2 activation by ⊿mSos1. BaF3-⊿mSosA6 cells were incubated in the culture medium with (○) or without (●) IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with human thrombin (1 U/ml) for indicated periods. The relative activity of ERK2 immunoprecipitated with a specific antibody (sc-154) is shown as mean±SD ($n=3$).

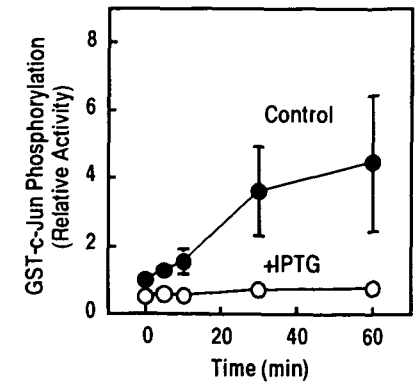


Fig. 6. **Effects of ⊿mSos1 on IL-3-induced JNK1 activation.** BaF3-⊿mSosA6 cells were incubated in the culture medium with (○) or without (●) IPTG (5 mM) for 24 h. Following starvation for 3 h, the cells were stimulated with mouse IL-3 (25 ng/ml) for indicated periods. The relative activity of JNK1 immunoprecipitated with a specific antibody (sc-474) is shown as mean±SD ($n=3$).

Shc as well as subsequent association of Shc with Grb2, events upstream of mSos, remained unaffected by induction of ⊿mSos1 (Fig. 4, C and D). To confirm the specificity of the ⊿mSos1 action, we further tested whether ⊿mSos1 displays an inhibitory effect on the JAK2 tyrosine kinase, which is presumably responsible for tyrosine phosphorylation of the β_c subunit of the IL-3 receptor and the following signal transduction events including Ras activation (*44, 45*). As expected, ⊿mSos1 did not affect tyrosine phosphorylation of JAK2 upon IL-3 stimulation (data not shown).

To further assess the inhibitory effect of ⊿mSos1 on the Ras pathway, the kinase activity of ERK2 was measured in the presence or absence of IPTG-induced ⊿mSos1. As shown in Fig. 5A, IPTG-induced expression of ⊿mSos1 rendered ERK2 insensitive to IL-3 stimulation, which causes remarkable enhancement of ERK2 activity through activation of Ras in the absence of ⊿mSos1. ERK2 activation upon thrombin treatment was prevented by ⊿mSos1 up to approximately 40% of the maximal level as illustrated in Fig. 5B. Since both Ras-dependent and independent signaling pathways leading to ERK2 activation are stimulated by thrombin (Fig. 2B), the ⊿mSos1-insensitive activation is thought to be mediated by the Ras-independent pathway. Taken together, the results implicate mSos as a critical component for IL-3- as well as thrombin-induced Ras-dependent ERK2 activation.

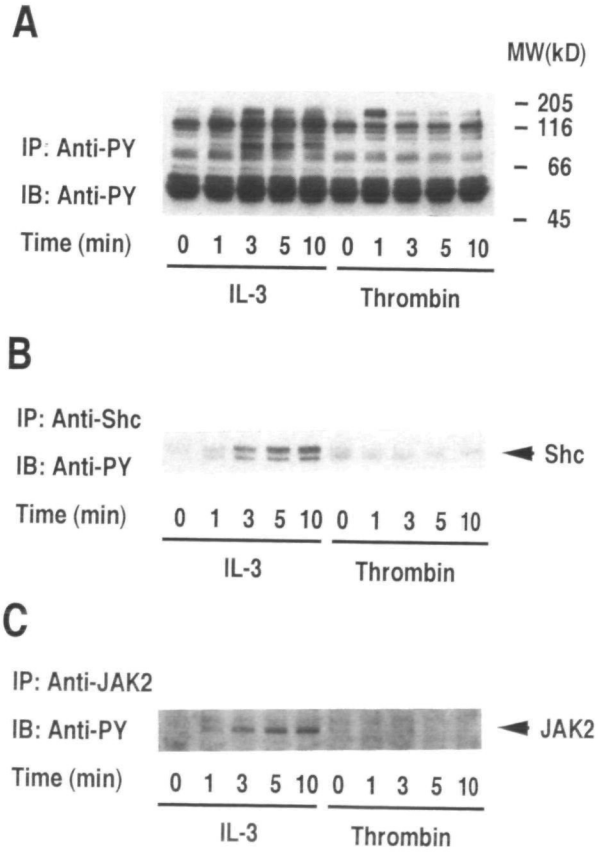


Fig. 7. **Tyrosine phosphorylation of cellular proteins upon IL-3 or thrombin stimulation.** (A) Tyrosine phosphorylation of total cellular proteins in IL-3- or thrombin-stimulated BaF3 cells. Following starvation for 3 h, BaF3 cells were stimulated with mouse IL-3 (25 ng/ml) or human thrombin (1 U/ml) for indicated periods. Tyrosine-phosphorylated proteins were detected by immunoprecipitation with an anti-phosphotyrosine antibody (05-321) and subsequent immunoblotting with the same antibody. (B) Tyrosine phosphorylation of Shc in IL-3- or thrombin-stimulated BaF3 cells. Following starvation for 3 h, BaF3 cells were stimulated with mouse IL-3 (25 ng/ml) or human thrombin (1 U/ml) for indicated periods. Tyrosine-phosphorylation of Shc was detected by immunoprecipitation with an anti-Shc antibody (06-203) and subsequent immunoblotting with an anti-phosphotyrosine antibody (05-321). (C) Tyrosine phosphorylation of JAK2 in IL-3- or thrombin-stimulated BaF3 cells. Following starvation for 3 h, BaF3 cells were stimulated with mouse IL-3 (25 ng/ml) or human thrombin (1 U/ml) for indicated periods. Tyrosine-phosphorylation of JAK2 was detected by immunoprecipitation with an anti-JAK2 antibody (sc-278) and subsequent immunoblotting with an anti-phosphotyrosine antibody (05-321).

Further evidence for the role of mSos in the Ras pathway is provided by the finding that IL-3-induced, Ras-dependent activation of JNK1, which was demonstrated in BaF3-derived cells (*43*), was also sensitive to the inhibitory effect of ⊿mSos1 (Fig. 6). On the other hand, thrombin-induced activation of JNK1 was totally insensitive to the interfering effect of Ras(S17N) in BaF3-N6 cells, suggesting a Ras-independent mechanism (data not shown). Collectively, activities of all protein kinases tested in the current work, which are regulated by Ras, failed to be activated subsequent to IL-3 and thrombin stimulation in the presence of ⊿mSos1, suggesting that mSos is indispensable for the signal transduction to stimulate the biochemical activity of Ras in response to these stimuli.

To gain further insight into the mechanistic role of mSos in IL-3 as well as thrombin signaling pathways, involvement of tyrosine kinases and adaptor proteins was explored. Unlike the remarkable induction of diverse tyrosine-phosphorylated proteins following IL-3 treatment, only a rapid and transient tyrosine-phosphorylation of a protein with molecular mass of approximately 150 kDa was detectable upon thrombin stimulation (Fig. 7A). In particular, tyrosine phosphorylation of Shc and JAK2 was not observed in response to thrombin (Fig. 7, B and C), whereas IL-3 potently induced these events. Hence, tyrosine kinases have clearly distinct roles in IL-3 and thrombin signaling pathways, in contrast to their similar potency to stimulate Ras and downstream kinases as well as the critical role of mSos in common.

DISCUSSION

While the roles of molecules such as Grb2 and mSos in tyrosine kinase-type receptor-mediated pathways are well described, those of virtually all proteins implicated in transduction of Ras-activating signals through other types of receptors, including cytokine receptors, G protein-coupled receptors, and the T-cell receptor, remain to be solved. Tyrosine phosphorylation of diverse cellular proteins is considered important for cytokine signal transduction despite the absence of an intrinsic tyrosine kinase activity in cytokine receptor subunits. Cytoplasmic tyrosine kinases specific to individual receptors, particularly Src family and JAK family kinases, associate with the intracellular domain of the receptor, being activated in response to ligand stimulation (*27*). Furthermore, tyrosine phosphorylation of Shc and its association with the receptor and the adaptor Grb2, subsequent to stimulation with multiple cytokines including IL-2, IL-3, and erythropoietin, have been demonstrated (*9, 28-35*). Although these results

suggest a mechanism similar to tyrosine kinase receptor-mediated Ras activation, it remains to be clarified whether mSos is crucial for cytokine receptor-mediated signaling.

Likewise, the role of mSos in G protein-coupled receptor-mediated signaling is still incompletely understood, although studies showing the involvement of mSos in Ras activation through certain types of G protein-coupled receptors were undertaken by transient expression in COS cells. We employed an inducible expression system to evaluate the role of mSos in cytokine receptor as well as G protein-coupled receptor-dependent activation of Ras in a hematopoietic cell line, BaF3. Thrombin and IL-3 seem to exert different biological functions, because thrombin displayed no growth-promoting effect on BaF3 cells, while IL-3 fully stimulates continuous cell growth (data not shown). Thrombin-dependent ERK2 activation was entirely sensitive to PTX pretreatment in BaF3 cells (Fig. 1C), suggesting the involvement of G_i family G proteins. Subsequent to G protein stimulation, the thrombin signal is mediated through both Ras-dependent and independent pathways as revealed by a partial inhibition of ERK2 activation by dominant-negative Ras(S17N) (Fig. 2B). Ras function is reported to be crucial for G protein $\beta\gamma$ subunit-mediated ERK/MAPK activation (*15, 17*), raising the possibility that Ras-dependent thrombin signaling is triggered by $\beta\gamma$ subunits also in the BaF3 cell line. On the other hand, the Ras-independent pathway may be directed by a member of G_i family α subunits. In contrast to BaF3 cells, both PTX-sensitive and insensitive pathways are responsible for ERK/MAPK activation mediated by the thrombin receptor in lung fibroblasts (*46*). Although it remains obscure whether Ras is implicated in thrombin signaling in these cells, it is certain that different signaling pathways function downstream of the thrombin receptor in different types of cells.

We have shown that dominant-negative ⊿mSos1 exhibits potent inhibitory effects towards activation of the Ras pathway through IL-3 and thrombin receptors, suggesting a crucial role of mSos in both systems. However, signaling pathways linking the receptors and mSos may be largely different. Shc and JAK2 underwent remarkable tyrosine phosphorylation upon IL-3 stimulation, while thrombin did not seem to stimulate these events (Fig. 7, B and C). To date, the involvement of various kinds of tyrosine kinases, including Src, Pyk2, Lyn, Syk, and the epidermal growth factor receptor, in G protein-dependent stimulation of the Ras, MAPK pathway has been demonstrated (*47-50*). In CCL39 hamster fibroblasts, thrombin elicits tyrosine phosphorylation of Shc in a PTX-insensitive manner (*51*), which may be implicated in Ras activation, although thrombin stimulation of Ras is reported to be sensitive to PTX (*10*). Similarly, angiotensin II stimulation of cardiac myocytes causes the activation of Ras through rapid tyrosine phosphorylation of Shc and its association with Grb2 (*52*). This is also insensitive to PTX, unlike the case of BaF3 cells. In COS cells, G protein $\beta\gamma$ subunits rather than the α subunit are thought to be responsible for Ras activation through certain types of G protein-coupled receptors such as the m2 receptor. This pathway involves phosphatidylinositol 3-kinase γ, tyrosine kinases, adaptors including Shc and Grb2, and mSos (*15-17, 53*). Although the role of $\beta\gamma$ subunits in thrombin receptor-mediated Ras activation remains to be defined, signaling molecules upstream of mSos in BaF3 cells may be different from those implicated in the m2 receptor pathway. Additionally, involvement of a 100 kDa tyrosine-phosphorylated Grb2 SH3-binding protein, but not Src nor Shc, in PTX-sensitive lysophosphatidic acid and thrombin signaling was reported (*54*). Taken together, these findings suggest that the thrombin signal may cause translocation of mSos to the plasma membrane through a mechanism distinct from the case of IL-3.

In addition to mSos, $CDC25^{Mm}$/Ras-GRF (*55-58*) was identified as a Ras-specific GDP/GTP exchange factor in mammalian cells. $CDC25^{Mm}$/Ras-GRF seems to be responsible for neuronal cell-specific signal transduction because of its exclusive expression in the brain. In fact, it has been proposed that calcium activation of Ras is mediated by $CDC25^{Mm}$/Ras-GRF in neuronal cells (*59*). Also, $CDC25^{Mm}$/Ras-GRF has been reported to be implicated in Ras activation through G protein-coupled receptors in a phosphorylation-dependent manner (*60*). A recently identified member of the $CDC25^{Mm}$/Ras-GRF family, Ras-GRF2, is widely expressed in mammalian tissues (*61*). This protein may also participate in G protein-coupled receptor signaling. Considering our observation that mSos is crucial for thrombin-dependent signaling to Ras in BaF3 cells, the mechanism for G protein-coupled receptor stimulation of the Ras pathway may differ depending on cell types.

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