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Title: STAP-2 regulates c-Fms/M-CSF receptor signaling in murine macrophage

Raw 264.7 cells

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Running title: STAP-2 regulates c-Fms/M-CSFR signaling

Abstract

Signal transducing adaptor protein-2 (STAP-2) is a recently identified adaptor protein as a c-Fms/M-CSF receptor-interacting protein and constitutively expressed in macrophages. Our previous studies also revealed that STAP-2 binds to MyD88 and IKK- α/β , and modulates NF- κ B signaling in macrophages. In the present study, we examined physiological roles of the interaction between STAP-2 and c-Fms in Raw264.7 macrophage cells. Our immunoprecipitation has revealed that c-Fms directly interacts with the PH domain of STAP-2 independently on M-CSF-stimulation. Ectopic expression of STAP-2 markedly suppressed M-CSF-induced tyrosine phosphorylation of c-Fms as well as activation of Akt and extracellular signal regulated kinase. In addition, Raw264.7 cells over-expressing STAP-2 showed impaired migration in response to M-CSF and wound-healing process. Taken together, our findings demonstrate that STAP-2 directly binds to c-Fms and interferes with the PI3K signaling, which leads to macrophage motility, in Raw 264.7 cells.

Introduction

Colony-stimulating factors (CSFs) are involved in normal progress of hematopoietic progenitor cells into terminal differentiated state [1]. Macrophage-CSF (M-CSF/CSF-1) interacts with a single class of high-affinity receptors and controls the development of macrophages from myeloid progenitors [2, 3, 4]. The receptor for M-CSF is identical to the *c-fms* protooncogene product (c-Fms) and is endowed with intrinsic tyrosine kinase activity that is transmitted through M-CSF binding. M-CSF induces dimerization of its receptor, leading to autophosphorylation of a number of tyrosine residues through the cytoplasmic domain of the receptor that serve as binding sites for the Src homology 2 (SH2) domain-containing proteins, such as Src-family kinases, Grb2 and phosphatidylinositol 3-kinase (PI3K). These then facilitate activation of signal transduction pathways that control macrophage proliferation, differentiation and motility. For example, activation of ERK signaling promotes macrophage proliferation [5,6], while activation of PI3K enhances macrophage survival and migration via Akt [7,8,9]. In addition, two point mutations at 301 and 969 in the human *c-fms* gene have been known to confer constitutive tyrosine kinase activity on the receptor and activate its transforming potential [10].

We have cloned a novel adaptor molecule, signal transducing adaptor protein-2 (STAP-2) [11] whose human homolog is identical to a recently cloned adaptor molecule, BKS, a substrate of Brk (breast tumor kinase)[12]. STAP-2 contains an N-terminal Pleckstrin homology (PH) and a region distantly related to the SH2 domain as well as the proline-rich, tyrosine phosphorylation motifs and a YXXQ motif in its C-terminal region. STAP-2 is expressed in a variety of tissues and cells such as lymphocytes and macrophages as well as hepatocytes, and its abundant expression pattern has suggested wide range of functions in vivo. Indeed, we have reported that STAP-2 can modulate STAT3 and STAT5 transcriptional activity as well as Fc γ RI- and toll like receptor-mediated signals [11,13,14,15]. Importantly, STAP-2 is constitutively expressed in macrophages and binds to MyD88 and IKK- β [15]. Because our identification of STAP-2 was originally performed based on the capacity to recognize c-Fms, we here examined functional roles of STAP-2 in c-Fms-mediated signals.

In the present study, overexpression of STAP-2 in a macrophage cell line, Raw 264.7 resulted in impaired M-CSF-induced tyrosine phosphorylation of c-Fms and downstream signaling proteins including PI3K/Akt and Ras/ERK. Furthermore, we

showed that c-Fms directly interacts with the PH domain of STAP-2. Finally, ectopically expressed STAP-2 resulted in impaired M-CSF-induced migration and wound-healing process. These results indicate that STAP-2 is a negative modulator in c-Fms-mediated signaling required for macrophage motility.

Materials and Methods

Reagents and antibodies

Recombinant human M-CSF was a kind gift from Morinaga Milk Industry Co., Ltd (Tokyo, Japan). Expression vectors, c-Fms, epitope-tagged STAP-2 and its YF (substitution of Tyr to Phe) mutants were described previously [11,13,16]. Antibody against c-Fms, ERK1, and GST antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Myc, anti-phosphotyrosine monoclonal (PY20), and anti-Actin antibodies were purchased from Sigma (St Louis, MO, USA). Antibodies against Akt, phospho-Akt, phospho-ERK, and phospho-c-Fms were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphoSTAP-2 Tyr250 (anti-pSTASP-2 Tyr250) and anti-mouse STAP-2 antibodies were prepared as described previously [16].

Cell culture, transfection and cell growth assays

Murine macrophage cell line, Raw264.7, was cultured in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Stable Raw264.7 cell transformants expressing empty vector, STAP-2 WT or STAP-2 mutants (Raw/STAP-2 Δ PH, Raw/STAP-2 Δ SH2 or Raw/STAP-2 Δ C) were prepared as described previously [15]. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol [13].

Cell migration assay

In vitro macrophage migration assay was conducted as previously described [17]. Briefly, the transwell filters (8 μ M pore filter; BD Falcon, Franklin lakes, NJ, USA) were placed in the lower chamber containing 500 μ l complete medium with or without M-CSF (100 ng/ml). Raw 264.7 cells (2.5×10^5) were resuspended in 100 μ l DMEM and allowed to migrate toward the underside of the top chamber. After 24 hrs of incubation at 37°C, the numbers of migrated cells of the lower chamber were counted with a phase contrast microscope.

Wound-healing assay

Wound-healing assay was performed as described previously [17]. The assay was conducted in 6-well tissue-culture plates. Raw 264.7 cells were cultured as a confluent monolayer, an artificial wound was created by scraping with a pipette tip, and cells migrating into open space were monitored microscopically. Photographs were taken immediately and at various intervals.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously [13]. Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride). An aliquot of total cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA, USA). The filters were then immunoblotted with the respective antibody.

Results and Discussion

Molecular interactions between STAP-2 and c-Fms

We originally identified STAP-2 as a c-Fms-interacting protein with a yeast two-hybrid system [11]. To verify whether STAP-2 is capable of interacting with c-Fms in mammalian cells, Myc-tagged STAP-2 was transiently expressed together with c-Fms in 293T cells. The transfectants were lysed, immunoprecipitated with an anti-c-Fms antibody, and then immunoblotted with an anti-Myc antibody. As shown in Fig. 1A, c-Fms was co-immunoprecipitated with Myc-STAP-2. Notably, the co-immunoprecipitation of c-Fms with Myc-STAP-2 was not dependent on M-CSF stimulation (Fig. 1A). As over-expression of c-Fms in 293T cells may have led to ligand-independent activation of the receptor, the ability of endogenous STAP-2 to bind to endogenous c-Fms in Raw 264.7 cells was also tested. The immunoprecipitates with anti-c-Fms antibody, but not those with control antibody, contained endogenous STAP-2 proteins, and similar amount of STAP-2 protein was contained in the cell lysates from Raw264.7 cells with or without M-CSF-stimulation (Fig. 1B). Therefore, association of STAP-2 with c-Fms was always observed and ligand-activation of c-Fms was not

required.

In order to identify which domain(s) of STAP-2 mediated its interaction with c-Fms, various mutants of STAP-2 were created (Fig. 1C). The ability of c-Fms to bind to the various GST-fused STAP-2 mutants was tested by co-expressing the proteins in 293T cells and then immunoprecipitating using anti-c-Fms antibody. Western blotting with an anti-GST antibody revealed that the immunoprecipitates with anti-c-Fms antibody predominantly contained the PH domain, but not the C-terminal domain of STAP-2, while the SH2 domain of STAP-2 weakly interacted with c-Fms (Fig. 1D). Therefore, the association of STAP-2 with c-Fms was mainly mediated by the PH domain of STAP-2.

STAP-2 contains several domains that have the potential to mediate protein–protein interaction. Indeed, STAP-2 associates with STAT3 via its YXXQ motif and with STAT5 via its PH and SH2-like domains. STAP-2 also binds MyD88 and IKK- α via its SH2-like domains. With regard to the association between STAP-2 and c-Fms, the PH domain of STAP-2 is important. Thus, many signaling molecules is likely to share STAP-2 *in vivo*. In addition, the binding of STAP-2 to c-Fms did not require M-CSF-stimulation, suggesting that STAP-2 might influence early step of c-Fms-mediated signals. Activation-independent association of STAP-2 is also observed in the cases of

IKK- α/β and MyD88 [15]. In contrast, association of STAP-2 with phospholipase C- β is dependent on Fc γ R activation [14].

M-CSF-induced phosphorylation on tyrosine-250 of STAP-2 by c-Fms

The physical interactions between STAP-2 and c-Fms clued us to investigate tyrosine-phosphorylation of STAP-2 by c-Fms. As shown in Fig. 2A, co-expression of STAP-2 with c-Fms demonstrated the marked tyrosine phosphorylation of STAP-2 after stimulation with M-CSF. We previously identified four potential phosphorylation sites of STAP-2 via substitution of tyrosine residues with phenylalanine (Fig. 2B). We then tried to determine the tyrosine-phosphorylation sites of STAP-2 by c-Fms. Myc-tagged wild-type STAP-2 (STAP-2 WT) or a series of STAP-2 YF mutants were co-expressed together with or without c-Fms in 293T cells. The expressed STAP-2 WT and YF mutant proteins were immunoblotted with anti-phosphotyrosine (PY), anti-pSTAP-2 Tyr-250 or anti-Myc antibodies. As shown in Fig. 2C, the STAP-2 WT and YF mutant proteins, with or without c-Fms co-expression, were expressed at equivalent protein levels. STAP-2 WT was tyrosin-phosphorylated after M-CSF-stimulation when expressed with c-Fms. However, STAP-2 Y250F showed remarkably diminished

tyrosine-phosphorylation and a slightly faster mobility than STAP-2 WT. This mobility shift of STAP-2 Y250F may be a result of loss of phosphorylation. Indeed, the lack of Tyr250 phosphorylation was confirmed by the western blot with an anti-pSTAP-2 Tyr-250 antibody. In addition, tyrosine-phosphorylation of STAP-2 Y310F was also diminished slightly. Therefore, Tyr-250 of STAP-2 is a major phosphorylation site by c-Fms, while c-Fms phosphorylated Tyr-310 of STAP-2 weakly.

Previous studies have demonstrated that STAP-2 is tyrosine-phosphorylated by several protein tyrosine kinases, including Brk, v-src, Jak2, Syk and EGFR [11,12,16]. Similarly, tyrosine-phosphorylation of STAP-2 was induced by c-Fms after M-CSF-stimulation. Up to date, we have shown that leukemia inhibitory factor-induced phosphorylation of STAP-2 on Tyr-250 is involved in its STAT3-enhancing activity [16]. Therefore, c-Fms-induced tyrosine-phosphorylation of STAP-2 may play a role in activation of the downstream signaling molecules as the case of STAT3.

STAP-2 suppresses M-CSF-induced tyrosine-phosphorylation of c-Fms and Akt activation in Raw 264.7 cells

To assess the functional relevance between STAP-2 and c-Fms-mediated signaling,

we established stable transformants expressing STAP-2 in Raw264.7 cells [15], and used them to evaluate the effect of STAP-2 on the M-CSF-induced signaling. STAP-2 expression of each Raw264.7 clone was confirmed with western blot (data not shown). Ectopic expression of STAP-2 did not influence on cell growth or cell viability in Raw264.7 cells (data not shown). First, we examined the effects of STAP-2 on M-CSF-induced tyrosine phosphorylation of c-Fms in Raw 264.7 cells. As shown in Fig. 3, after stimulation of M-CSF, tyrosine phosphorylation of c-Fms was induced in Raw/pcDNA3 cells. However, a significant reduction of tyrosine phosphorylation of c-Fms was observed in Raw/STAP-2 cells. In addition, ectopic expression of STAP-2 also affected M-CSF-induced Akt activation, which lies downstream signaling pathways of PI3K. M-CSF-induced ERK activation was also affected by expression of STAP-2. Therefore, STAP-2 regulates M-CSF-mediated both PI3K/Akt and Ras/ERK signaling pathways in macrophages.

M-CSF induces recruitment of the p85 subunit of PI3K to c-Fms and Gab2, resulting in rapid activation of the PI3K/Akt and Ras/ERK signaling pathways [5-9]. Previous studies using pharmacologic inhibitors of PI3K, such as LY294002 [9] as well as injection of antibodies to inhibit the function of the catalytic subunits of PI3K [18], have suggested a role of the PI3K pathway in macrophage migration. Thus, STAP-2 may

regulate M-CSF-induced macrophage migration via the suppression of the PI3K pathway.

STAP-2 suppresses M-CSF-induced macrophage migration in Raw 264.7 cells

Because STAP-2 suppressed M-CSF-induced PI3K/Akt activation, we examined the effect of STAP-2 on migration of Raw 264.7 transfectants with an in vitro migration assay. As shown in Fig. 4A, ectopic expression of STAP-2 results in a significant reduction in directed migration of Raw264.7 cells in response to M-CSF. Macrophages are well known to play a significant role in wound-healing [19]. To assess the functional domains of STAP-2 on M-CSF-induced cell migration, we used stable transformants expressing STAP-2 deletion mutants (Fig. 4B) in Raw264.7 cells. As shown in Fig. 4C, reduction of M-CSF-induced cell migration was observed in Raw/STAP-2 Full, Raw/STAP-2 Δ SH2 and Raw/STAP-2 Δ C cells compared with Raw/pcDNA3 cells. However, Raw/STAP-2 Δ PH cells did not show any reduction of M-CSF-induced cell migration, suggesting that the PH domain of STAP-2 plays an important role in modulating M-CSF-induced migration in Raw 264.7 cells. During the process of inflammation, macrophages migrate and extravagate into numerous tissues. However,

the signaling pathways that regulate the migration of macrophages are poorly understood. Using bone marrow-derived macrophages lacking PI3K p85 α -subunit, PI3K p85 α has been demonstrated to be crucial for migration in a wound-healing assay compared with wild-type controls [20]. Interestingly, as shown in Fig. 4D, ectopic expression of STAP-2 in Raw 264.7 cells also resulted in reduced migration in an in vitro wound-healing assay. Therefore, STAP-2 plays an important role in the regulation of macrophage migration.

Concluding remarks

Our most important finding is that STAP-2 directly binds to c-Fms and suppresses tyrosine-phosphorylation of c-Fms and its downstream signaling events including PI3K/Akt and Ras/ERK. The suppression of M-CSF-induced signals resulted in decrease of macrophage migration capacity. Taken together with the fact that STAP-2 mRNA is induced by the stimuli with lipopolysaccharide or IL-6, STAP-2 might act to keep macrophages at inflammatory sites. Importantly, we previously reported that STAP-2 enhances TLR4-mediated NF- κ B activation in macrophages and IL-6-induced acute phase gene expression in hepatocytes. Thus, STAP-2 is likely to function to limit infections in vivo.

To maintain macrophage homeostasis, the c-Fms-mediated signaling pathway must be tightly regulated. SOCS-1 and Dok-2 were demonstrated to play roles in the negative regulation of c-Fms signaling [21,22]. SOCS-1 partially suppresses M-CSF signaling by competing with Grb2, phospholipase C- β 2 or PI3K for binding to Tyr-697 and/or Tyr-721 in the activated c-Fms [22]. Dok-2 regulates c-Fms signaling through inhibition of the phosphorylation of Shc by Src-family kinases, which resulted in impaired inducible gene expression [21]. Another key protein involved in the negative regulation of signaling by c-Fms is c-Cbl, an E3-ubiquitin ligase [23]. c-Cbl terminates c-Fms signaling by binding to the phosphorylated Tyr-973 in the C-terminus of the activated receptor and recruiting E2-ubiquitin ligases to the receptor [24,25]. Subsequent polyubiquitination of c-Fms by the E2-ubiquitin ligases targets it for internalization and degradation [23]. Importantly, there might be the possibility that STAP-2 also participates in c-Cbl-mediated regulation of c-Fms signaling because c-Cbl was found to interact with STAP-2 in 293T cells (Y. Sekine and T. Matsuda, unpublished data). Thus, STAP-2 has direct and indirect regulation in c-Fms-mediated signals.

In the present paper, we have described evidence that STAP-2 can directly interact with c-Fms and regulates c-Fms-mediated signaling pathways, especially cell migration. Further detailed studies are necessary to elucidate the molecular mechanisms of

suppression in macrophage migration by STAP-2. However, our proposed signaling pathway concerning STAP-2 will provide new insights toward understanding the molecular mechanisms of macrophage homeostasis. In addition, our data suggest the possibility that STAP-2 can be considered as a novel candidate for therapeutic drug development to modulate wound-healing process and inflammation.

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Figure legends

Fig. 1. Molecular interactions between STAP-2 and c-Fms

(A) 293T cells (1×10^7 cells) were transfected with c-Fms (3 μ g) together with or without Myc-STAP-2 (5 μ g). At 36 hrs after transfection, the cells were stimulated with M-CSF (100 ng/ml) for the indicated periods. The cells were lysed and immunoprecipitated with anti-c-Fms antibody and blotted with anti-Myc, anti-c-Fms or anti-pc-Fms antibody (upper panels). Total cell lysates (1%) were blotted with anti-Myc, anti-pc-Fms or anti-c-Fms antibody (lower panels). (B) Murine macrophage Raw 264.7 cells (3×10^7) were stimulated with M-CSF (100 ng/ml) for 5 min or left unstimulated. The cells were lysed and immunoprecipitated with control IgG or anti-c-Fms antibody and blotted with anti-STAP-2 (upper panel) or anti-c-Fms antibody (lower panel). Total cell lysates (1%) were blotted with anti-pc-Fms, anti-c-Fms or anti-STAP-2 antibody (right panels). (C) Domain structure of STAP-2 and GST-fused mutant fragments are schematically shown. (D) 293T cells (1×10^7) were transfected with c-Fms (3 μ g) together without or with GST-fused STAP-2 and its deletion mutants (7.5 μ g). At 36 hrs after transfection, the cells were lysed and immunoprecipitated with anti-c-Fms antibody and blotted with anti-GST or anti-c-Fms antibody (left panels). Total cell extracts were blotted with anti-GST antibody (right panel).

Fig. 2. Phosphorylation of Tyr-250 in STAP-2 by c-Fms

(A) 293T cells in a 12-well plate were transfected with or without Myc-tagged STAP-2 WT (0.5 μ g) and/or c-Fms (0.5 μ g). At 36 hrs after transfection, the cells were stimulated with M-CSF (100 ng/ ml) for 5 min or left unstimulated and lysed. Total cell lysates (1%) were blotted with anti-PY, anti-Myc, anti-pc-Fms or anti-c-Fms antibody. (B) Domain structure of STAP-2 is schematically shown. Four predicted tyrosine residues are also shown. (C) 293T cells in a 12-well plate were transfected with or without Myc-tagged STAP-2 WT (0.5 μ g) or STAP-2 YF mutants (0.5 μ g) and/or c-Fms (0.5 μ g). At 36 hrs after transfection, the cells were stimulated with M-CSF (100 ng/ ml) for 5 min and lysed. Total cell lysates (1%) were blotted with anti-PY, anti-pSTAP-2 Tyr-250, anti-Myc, anti-pc-Fms or anti-c-Fms antibody.

Fig. 3. STAP-2 suppresses M-CSF-induced tyrosine-phosphorylation of c-Fms and Akt activation in Raw 264.7 cells

(A) Raw/pcDNA3 or Raw/STAP-2 cells in a 12 well-plate were stimulated with or without M-CSF (100 ng/ml) for the indicated periods. The cells were lysed, and then immunoblotted with anti-pc-Fms, anti-c-Fms, anti-pAkt, anti-Akt, anti-pERK, anti-ERK,

anti-Myc or anti-Actin antibody. (B) Densitometric quantification of the above results was also shown. Relative intensity of pc-Fms, pERK or pAkt was normalized to the respective protein of the same sample.

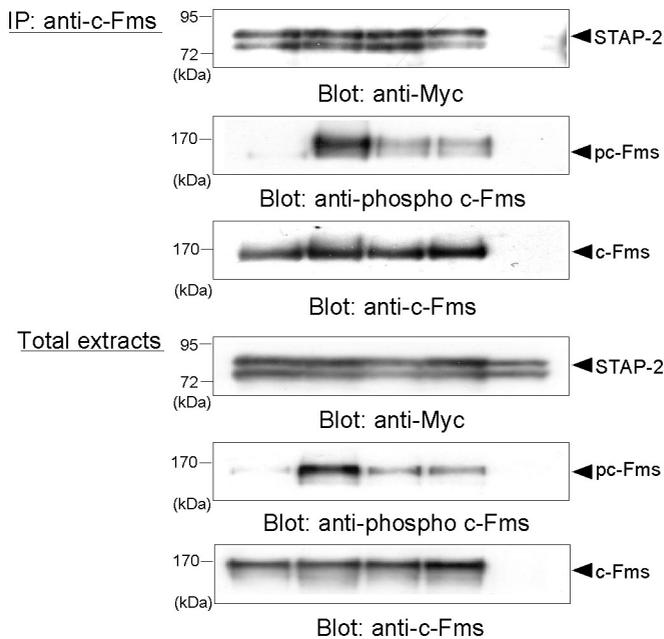
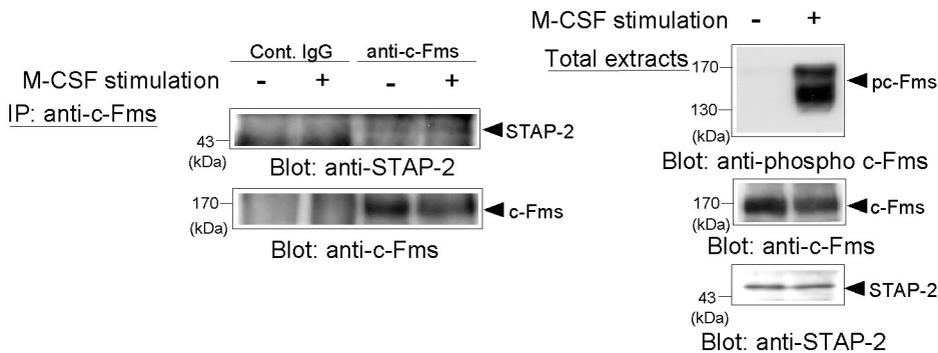
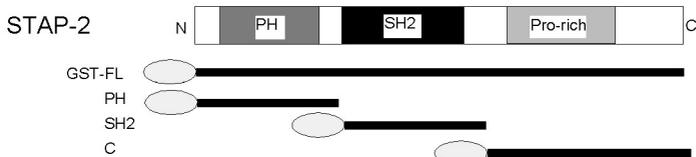
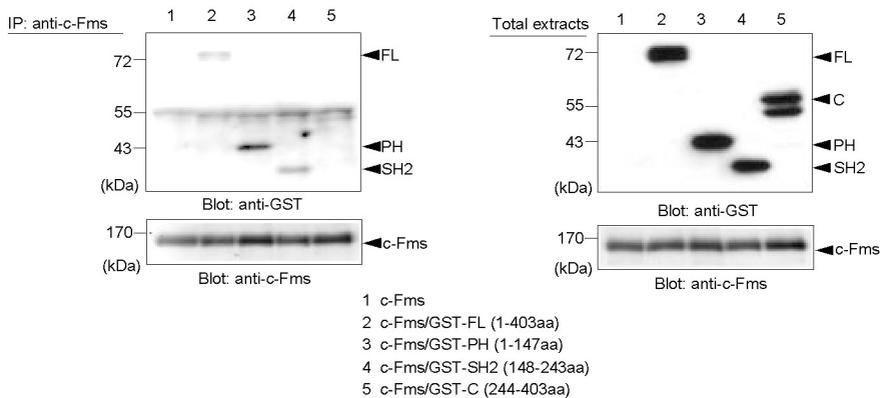
Fig. 4. STAP-2 suppresses M-CSF-induced macrophage migration in Raw 264.7 cells

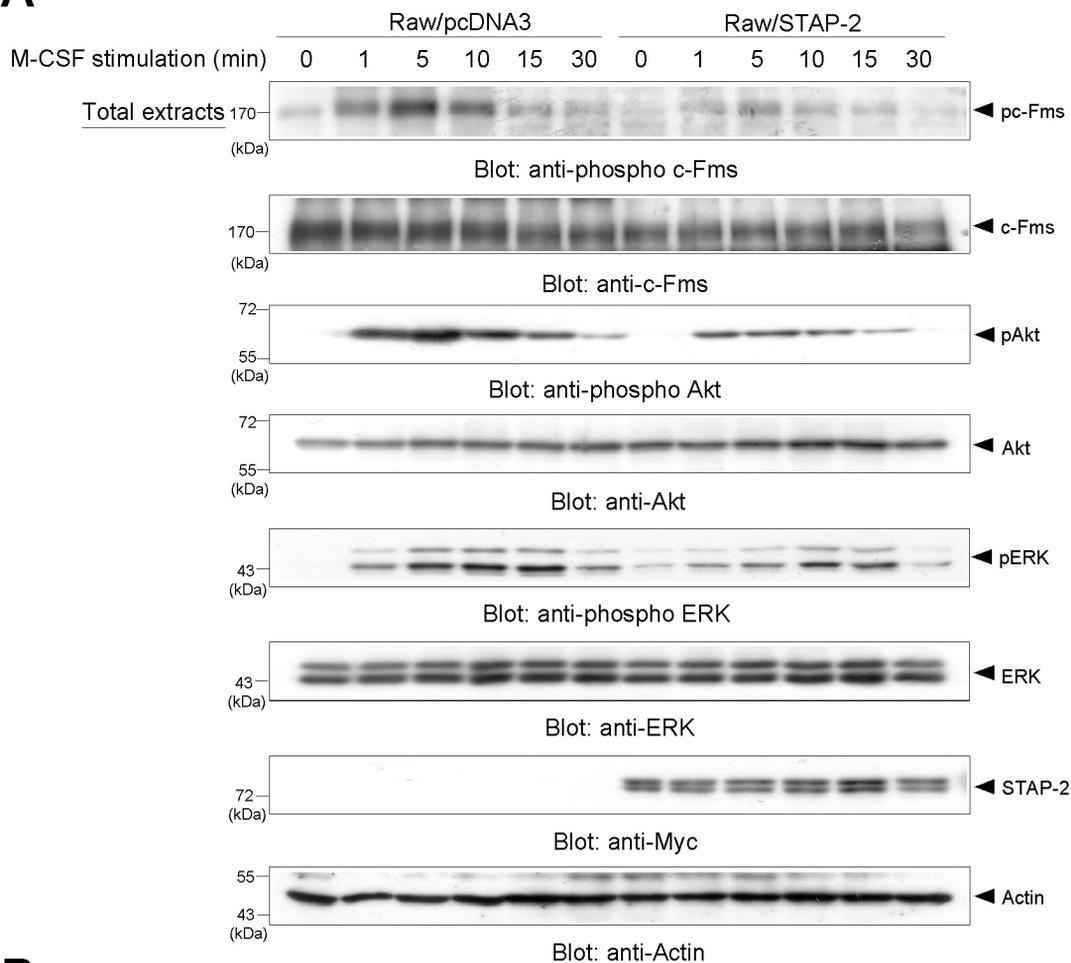
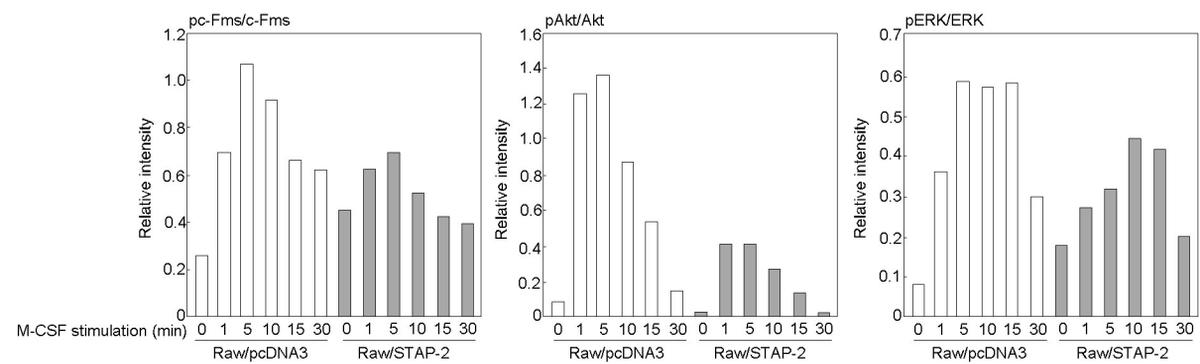
(A) Raw/pcDNA3 or Raw/STAP-2 cells (2×10^5) were subjected to an in vitro migration assay on transwell membranes. Cell migration was performed either in the absence or presence of M-CSF (50 or 100 ng/ml). Cell migration is expressed as the average number of cells migrated. Figure shows the average number of cells migrated \pm SD. Similar results were observed in three independent experiments. (B) Domain structure of STAP-2 and its deletion mutants are schematically shown. (C) Raw/pcDNA3, Raw/STAP-2 WT, Raw/STAP-2 Δ PH, Raw/STAP-2 Δ SH2 or Raw/STAP-2 Δ C cells (2×10^5) were subjected to an in vitro migration assay on transwell membranes. Cell migration was performed either in the absence or presence of M-CSF (100 ng/ml). Cell migration is expressed as average number of cells migrated. Figure shows fold induction relative to Raw/pcDNA3 untreated with M-CSF for each \pm SD. Similar results were observed in three independent experiments. (D) Raw/pcDNA3 or Raw/STAP-2 cells were cultured in a 6-well plate. An artificial wound was created in

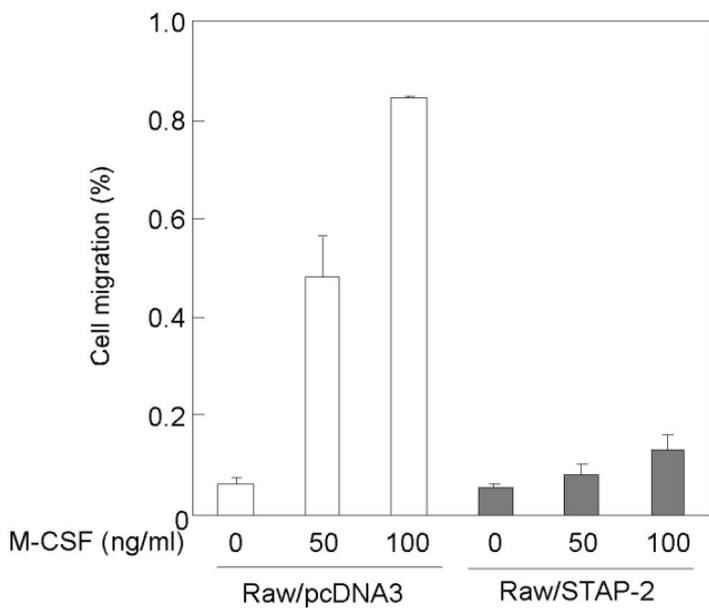
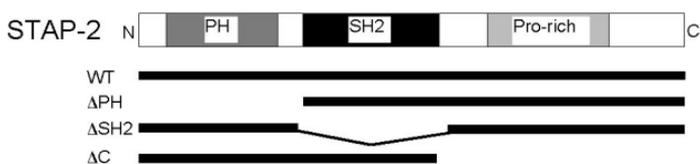
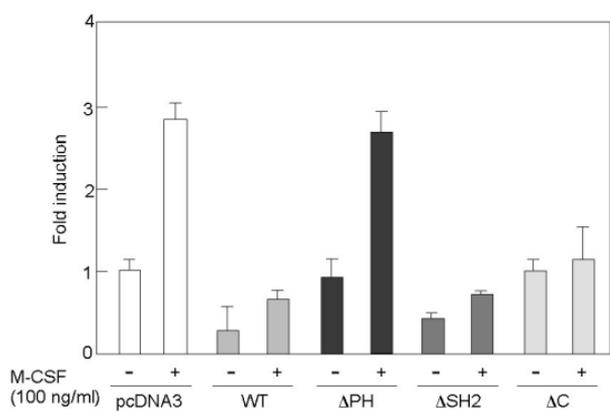
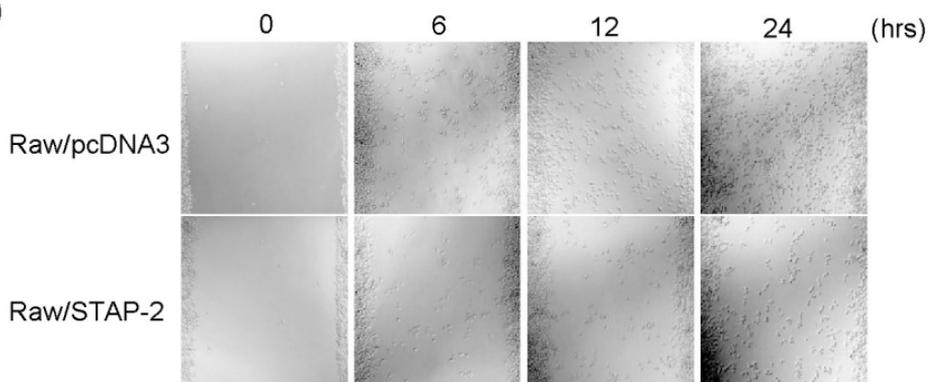
the macrophage monolayer using a pipette tip. Photographs were taken immediately and again at indicated time periods after creating the wound. Data are from one representative experiment. Similar results were obtained in three independent experiments.

A

c-Fms	+	+	+	+	-
Myc-STAP-2	+	+	+	+	+
M-CSF stimulation (min)	0	5	15	30	0

**B****C****D****Figure 1**

A**B****Figure 3**

A**B****C****D****Figure 4**