

# The Scaffold Protein c-Jun NH2-Terminal Kinase-associated Leucine Zipper Protein Regulates Cell Migration through Interaction with the G Protein G13

メタデータ	言語: eng 出版者: 公開日: 2017-10-05 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	<a href="https://doi.org/10.24517/00027541">https://doi.org/10.24517/00027541</a>

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 International License.



# The Scaffold Protein c-Jun NH<sub>2</sub>-Terminal Kinase-associated Leucine Zipper Protein Regulates Cell Migration through Interaction with the G Protein G<sub>α13</sub>

Davaakhuu Gantulga<sup>1</sup>, Baljinnyam Tuvshintugs<sup>1</sup>, Yoshio Endo<sup>2</sup>, Takahisa Takino<sup>2</sup>, Hiroshi Sato<sup>2</sup>, Seishi Murakami<sup>1</sup> and Katsuji Yoshioka<sup>1,\*</sup>

<sup>1</sup>Division of Molecular Cell Signaling, Department of Molecular and Cellular Biology; and <sup>2</sup>Division of Molecular Virology and Oncology, Department of Molecular Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, 920-0934, Japan

Received July 11, 2008; accepted September 9, 2008; published online September 30, 2008

**Scaffold proteins for MAP kinase (MAPK) signalling modules play an important role in the specific and efficient signal transduction of the relevant MAPK cascades. Here, we investigated the function of the scaffolding protein c-Jun NH<sub>2</sub>-terminal kinase (JNK)-associated leucine zipper protein (JLP) by depleting it in cultured cells using a short hairpin RNA (shRNA) against human JLP. HeLa and DLD-1 cells stably expressing the shRNA showed a defect in cell migration. The re-expression of full-length shRNA-resistant mouse JLP rescued the impaired cell migration of the JLP-depleted HeLa cells; whereas, a C-terminal deletion mutant of mouse JLP, which failed to bind the G protein G<sub>α13</sub>, showed little or no effect on the cell migration defect. Furthermore, although a constitutively active G<sub>α13</sub> enhanced the migration of control HeLa cells, the G<sub>α13</sub>-induced cell migration was significantly suppressed in the JLP-depleted HeLa cells. Taken together, these results suggest that JLP regulates cell migration through an interaction with G<sub>α13</sub>.**

**Key words:** cell migration, MAP kinase, p38, RNA interference, scaffold protein.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase; HEK, human embryonic kidney; JLP, JNK-associated leucine zipper protein; JNK, c-Jun NH<sub>2</sub>-terminal kinase; JSAP1, JNK/stress-activated protein kinase-associated protein 1; KD, knockdown; MAPK, MAP kinase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; shRNA, short hairpin RNA.

MAP kinase (MAPK) cascades transmit signals through the sequential phosphorylation and activation of three-tier kinase modules, consisting of MAPK kinase kinase, MAPK kinase and MAPK. Mammalian MAPK signalling pathways play a key role in multiple cellular functions, such as proliferation, migration and apoptosis (1, 2). The specificity of MAPK signalling is mediated, at least in part, by scaffold proteins. Scaffold proteins for MAPK cascades organize the MAPK signalling components into functional modules, thereby enabling the efficient activation of specific MAPK pathways (3–5). The scaffolding complexes also protect the signalling components within the relevant MAPK modules from undesired activation by other signalling molecules in cells.

c-Jun NH<sub>2</sub>-terminal kinase (JNK)-associated leucine zipper protein (JLP) was originally identified as a binding protein for the transcription factor Max, and further biochemical study indicated that JLP functions as a scaffold protein for the JNK and p38 MAPK signalling modules (6). JLP is broadly expressed, and is structurally related to JNK/stress-activated protein kinase-associated protein 1 (JSAP1 or JNK-interacting protein 3), a scaffold protein for JNK cascades (7–9).

Studies of JLP have mainly focused on identifying its interacting proteins, which include kinesin light chain 1 and G<sub>α13</sub>, the α-subunit of the heteromeric G<sub>13</sub> protein (10, 11). JLP has also been reported to play an important role in myogenesis by interacting with the cell-surface protein Cdo (12). To date, however, the physiological functions of JLP remain poorly understood.

Here, we established cultured cell lines expressing a short hairpin RNA (shRNA) against JLP, in which JLP expression was severely decreased, and we used these JLP knockdown (KD) cells to investigate the function of JLP.

## MATERIALS AND METHODS

**Cell Culture**—HeLa cells (human cervical carcinoma), DLD-1 cells (human colon carcinoma) and human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) in a 5% CO<sub>2</sub> atmosphere at 37°C. For immunostaining and cell migration assays, HeLa cells were cultured on plates coated with type I collagen (20 μg/ml; Nitta Gelatin, Osaka, Japan). For JNK and p38 activation, HeLa cells were stimulated with UV-C irradiation or anisomycin (3 μg/ml, 5–15 min). In the UV-C irradiation experiment, cells in a 35-mm dish were washed once with

\*To whom correspondence should be addressed. Tel/Fax: +81-76-234-4532, E-mail: katsuji@kenroku.kanazawa-u.ac.jp

phosphate-buffered saline (PBS), the PBS was removed, and the cells were exposed to UV-C light at 100 J/m<sup>2</sup>. The cells were then incubated in medium for 5–15 min.

**Expression Plasmids**—The following annealed oligonucleotides with hairpin sequences were inserted into *AgeI*/*EcoRI*-digested pMKO.1-puro (13) for the expression of shRNAs against JLP: JLP-shRNA1, forward, 5'-CCGG CTTGAGCTGAAAGCGAAAAACCTCGAGGTTTTTCGCT TTCAGCTCAAGTTTTTG-3' and reverse, 5'-AATTCAAA AACTTGAGCTGAAAGCGAAAAACCTCGAGGTTTTTCG CTTTCAGCTCAAG-3'; JLP-shRNA2, forward, 5'-CCGG TGAAGATGCAAGGCAAAAAGCCTCGAGGCTTTTTGC CTTGCATCTTCATTTTTG-3' and reverse, 5'-AATTCAAA AATGAAGATGCAAGGCAAAAAGCCTCGAGGCTTTTT GCCTTGCATCTTCA-3', where the underlined letters represent human JLP sequences (GenBank accession no. AF327452). To generate the plasmid vector pBabe-blast, the entire coding region of the blasticidin S-resistance gene was amplified by polymerase chain reaction (PCR) using pWZL-blast (14, 15) as a template, and the PCR product was replaced with the puromycin-resistance gene in pBabe-puro. The entire coding regions of Flag-tagged mouse Rac1 and mouse G<sub>v13</sub> (RegSeq accession no. NM\_009007 and NM\_010303, respectively) were obtained by PCR and subcloned into *Bam*HI/*Sal*I-digested pBabe-blast. To introduce nucleotide substitutions into cDNAs encoding G<sub>v13</sub> and the small GTPase Rac1, to create constitutively active forms of G<sub>v13</sub> (G<sub>v13</sub>Q226L) and Rac1 (Rac1G12V), site-directed mutagenesis was carried out by overlapping PCR as described previously (7). The region of the cytomegalovirus promoter in the plasmid vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) was amplified by PCR and inserted into pWZL-blast to generate pWZL-CMV-blast. The coding regions of Flag-tagged mouse full-length JLP (mJLP; residues 1–1307) and C-terminal truncated JLP (mJLPΔC; residues 1–1164) were obtained by PCR and subcloned into *Bam*HI/*Xho*I-digested pWZL-CMV-blast. The PCR product encoding the full-length wild-type G<sub>v13</sub> was inserted into *Kpn*I/*Xho*I-digested pcDNA3-Flag (7) to generate pcDNA3-Flag-G<sub>v13</sub>. The coding regions of the full-length mJLP (residues 1–1307) and mJLPΔC (residues 1–1164) were amplified by PCR and inserted into *Hind*III (filled in)/*Xho*I-digested pcDNA3.1-Hygro (Invitrogen) to generate pcDNA3-mJLP and pcDNA3-mJLPΔC, respectively. To generate expression plasmids for JLP glutathione S-transferase (GST) and His fusion proteins, the coding region of mJLP (residues 796–955) was amplified by PCR and subcloned into *Eco*RI/*Xho*I-digested pGEX4T-3 (GE Healthcare, Buckinghamshire, UK) or *Nhe*I/*Xho*I-digested pREST-B (Invitrogen). All the amplified sequences were verified by DNA sequencing.

**Antibodies**—The GST-mJLP (residues 796–955) and His-mJLP (residues 796–955) recombinant proteins expressed in *Escherichia coli* were purified with glutathione Sepharose (GE Healthcare) and Ni-NTA Agarose (Qiagen, Hilden, Germany) according to the manufacturer's instructions, respectively. The purified GST-JLP protein was used for antiserum production in Kbl:JW rabbits. The antiserum was absorbed serially on three columns: an immobilized NHS-activated Sepharose (GE Healthcare) column, which was made using whole

sonic extracts of *E. coli*, an immobilized GST-Sepharose column, and a column bearing immobilized Flag-tagged full-length JSAP1, which was obtained by transient expression in HEK293T cells. The flow-through fraction was further purified using an antigen-affinity column, the His-mJLP-Ni-NTA Agarose column, and used as the anti-JLP antibody in this study. Other primary antibodies were as follows: mouse monoclonal anti-Flag M2 (Sigma), rabbit polyclonal anti-actin (Sigma), mouse monoclonal anti- $\alpha$ -tubulin (Sigma), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; Dako, Glostrup, Denmark), mouse monoclonal and rabbit polyclonal anti-phospho-JNK (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-phospho-p38 (Cell Signaling Technology) and mouse monoclonal anti-paxillin (BD Biosciences, San Jose, CA, USA) antibodies. The antibody to actin (cat. no. A5060) shows a broad specificity with actin isoforms across a range of organisms. The monoclonal and polyclonal anti-phospho-JNK antibodies were used for immunoblotting and immunofluorescence, respectively. Secondary antibodies were goat Alexa fluor 488-conjugated anti-mouse IgG and goat Alexa fluor 546-conjugated anti-rabbit IgG antibodies (Invitrogen).

**Virus Production**—Recombinant retroviruses for the expression of JLP-shRNA1 and JLP-shRNA2 were prepared as described previously (16).

**Screening of Cell Lines**—HeLa cell lines stably expressing JLP-shRNA1 and JLP-shRNA2 were screened in the presence of puromycin as described previously (16). The selected cell populations were subjected to immunoblotting with the anti-JLP antibody. To obtain clones expressing JLP-shRNA1, HeLa cells (1 × 10<sup>6</sup> cells/60-mm dish) were infected with serially diluted recombinant virus for JLP-shRNA1, and the cells were selected as described previously (16). To establish cell lines stably expressing full-length mJLP, mJLαC, Flag-Rac1G12V or Flag-G<sub>v13</sub>Q226L in a JLP KD HeLa clone, the cells were split and plated onto 60-mm dishes (5 × 10<sup>5</sup> cells/dish) the day before transfection, and the relevant expression plasmids were introduced into the JLP KD HeLa cells with LipoTrust SR (Hokkaido System Science, Sapporo, Japan). Blasticidin S-resistant transfected cells were selected using blasticidin S (Kaken, Tokyo, Japan) at a concentration of 6 μg/ml.

**Immunoblotting and Immunofluorescence**—The preparation of total cell lysates and subsequent immunoblotting analyses were carried out as described previously (17). For cell fractionation experiments, cells were harvested by trypsin-ethylenediaminetetraacetic acid (EDTA), washed with PBS, resuspended in ice-cold hypotonic buffer containing 10 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, Protease Inhibitor Cocktail (Sigma), and Phosphatase Inhibitor Cocktail (Sigma), and homogenized with 20 strokes in a Dounce homogenizer (tight pestle). After centrifugation at 1,000g for 5 min at 4°C, the supernatant was used as the cytoplasmic fraction. The pellet was washed twice with ice-cold hypotonic buffer, resuspended in nuclear extraction buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, Protease Inhibitor Cocktail (Sigma), and

Phosphatase Inhibitor Cocktail (Sigma), and spun at 12,000g for 10 min at 4°C. The supernatant was used as the nuclear fraction. Immunofluorescence was performed as described previously (16). Images were captured with a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

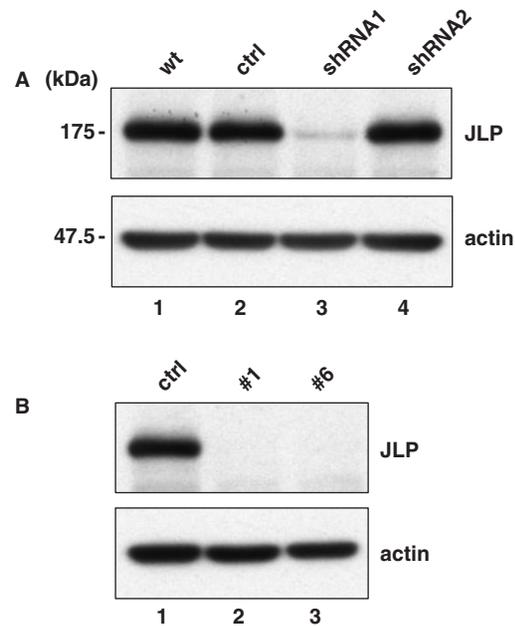
**Cell Migration Assay**—To measure the cell migration activity, Transwell and wound-healing assays were performed. The Transwell cell migration assay was carried out using a modified Boyden chamber (Corning, Corning, NY, USA) containing a gelatin-coated polycarbonate membrane filter (6.5-mm diameter, 8 μm pore size). The upper surface of the filter was coated with type I collagen. After serum starvation in DMEM containing 0.5% FBS for 12 h, cells (10,000 cells/100 μl) in DMEM with 0.5% FBS were added to the upper chamber. The lower chamber contained DMEM with either 10% FBS, or 0.5% FBS for the constitutively active Rac1- and G<sub>α13</sub>-induced migration assays, respectively. The cells were then incubated for 6 h at 37°C in 5% CO<sub>2</sub>. Migratory cells on the underside were detached by trypsinization and counted.

For the wound-healing assay, the cells were replated onto dishes coated with type I collagen at a saturation density ( $2.2 \times 10^6$  cells/35-mm dish) in the absence of serum, and incubated for 3 h. They were then scratched manually with a sterile pipette tip. After being washed with DMEM, the wounded regions were allowed to heal for 36 h in DMEM containing 10% FBS. The cell movements were monitored using a BZ-8000 microscope (Keyence, Osaka, Japan). Video images were collected at 1 h intervals for 36 h, and the image stacks were converted to QuickTime movies.

**Statistical Analysis**—Results are presented as the mean ± SD from the number of experiments indicated in the legends to Figs 3–5. Statistical differences were analysed using Student's *t*-test. Values of *P* < 0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

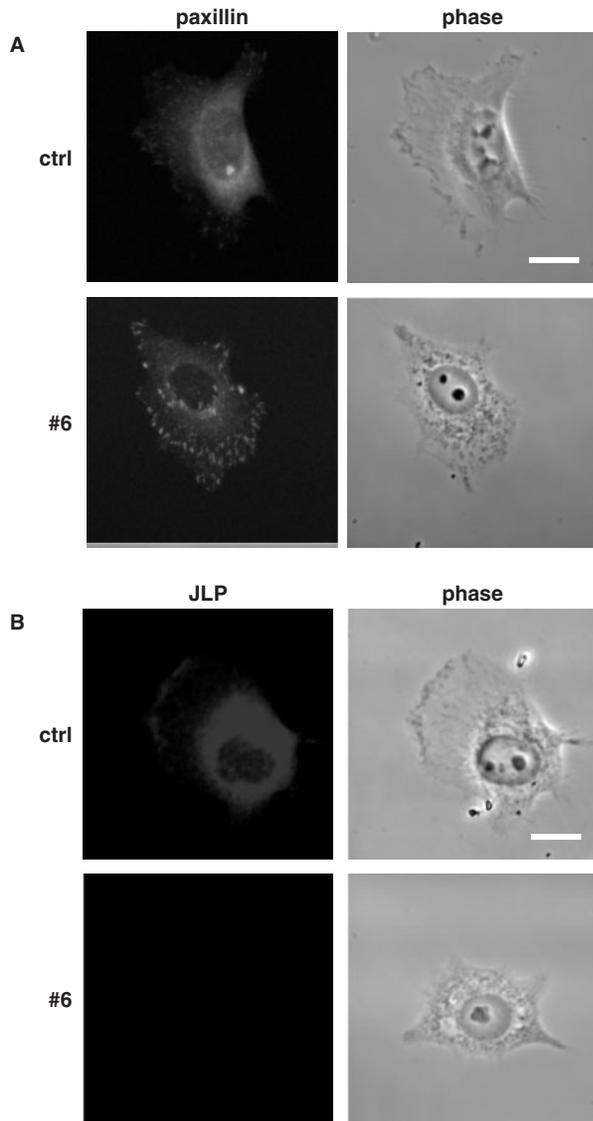
**Establishment of Stable HeLa Cell Lines Expressing an shRNA Against JLP**—To investigate the physiological functions of JLP, we used the RNA interference (RNAi) approach. We examined the effectiveness of two JLP-shRNAs: JLP-shRNA1 and -shRNA2 (see MATERIALS AND METHODS section). To this end, HeLa cells were infected with the respective recombinant retroviruses, selected in the presence of puromycin, and analysed by immunoblotting. The expression level of JLP protein in JLP-shRNA1-expressing cells was substantially decreased compared with the wild-type parent HeLa cells and control HeLa (HeLa<sup>ctrl</sup>) cells that had been infected with empty retrovirus (Fig. 1A; compare lanes 1, 2 and 3); whereas, JLP-shRNA2 had little effect on the expression of JLP (Fig. 1A, lane 4). Because the puromycin-resistant cells infected with JLP-shRNA1 could be heterogeneous with respect to JLP expression, we screened for independent clones that barely expressed any JLP protein. Indeed, the expression level of JLP protein was different among clones (data not shown), and almost no expression of JLP protein was observed in clones #1 and #6 (Fig. 1B, lanes 2 and 3).



**Fig. 1. Establishment of HeLa cell lines stably expressing shRNA against JLP.** (A) HeLa cells were infected independently with recombinant retrovirus for JLP-shRNA1 and -shRNA2, and selected with puromycin. Total cell lysates were prepared from the cells, and subjected to immunoblotting with an anti-JLP antibody. The wild-type (wt) parent HeLa cells, and the control (ctrl) HeLa cells, which were infected with empty retrovirus, were used as controls. Actin was used as a loading control. The positions of the protein size markers are indicated on the left. (B) The ctrl HeLa cells and JLP KD cells of clones #1 and #6, which were obtained after screening the pooled cells infected with JLP-shRNA1-expressing retrovirus, were subjected to immunoblotting with an anti-JLP antibody. Representative results are shown.

**Immunocytochemical Analysis of Control and JLP KD Cells**—We noticed that clones #1 and #6 were morphologically distinct from the HeLa<sup>ctrl</sup> cells; notably, the cell polarity seemed to be impaired in the JLP KD cells (data not shown). We therefore analysed the focal adhesion of the JLP KD cells by immunostaining. The shRNA-mediated KD of JLP resulted in increases in the number and size of paxillin-positive adhesions (Fig. 2A). Furthermore, we found a portion of the JLP protein in the HeLa<sup>ctrl</sup> cells to be localized to the leading edge (Fig. 2B). Taken together, these results indicate that JLP might play a role in cell migration.

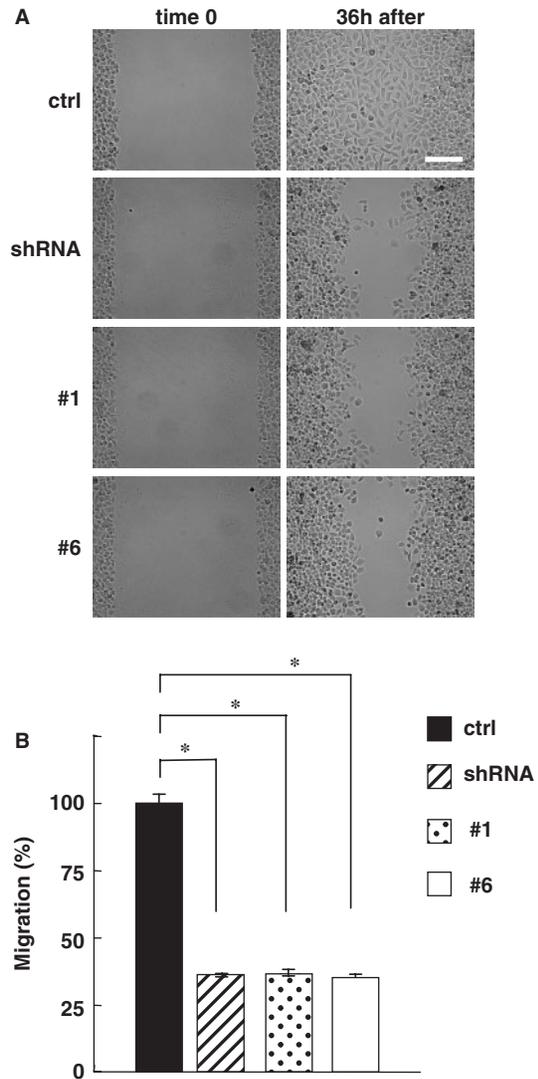
**Impairment of Cell Migration in JLP KD Cells**—We next examined the role of JLP in cell migration using wound-healing and Transwell assays (Fig. 3). HeLa<sup>ctrl</sup> cells migrating in scratch wound cultures showed wound closure by 36 h; whereas, the pooled HeLa cells expressing JLP-shRNA1 (HeLa<sup>JLP-shRNA1</sup>) and clones #1 and #6 showed retarded migration (Fig. 3A). We observed a similar cell-migration defect in a JLP KD DLD-1 cell line (data not shown), which had been established in the same way as the HeLa<sup>JLP-shRNA1</sup> cell line. For quantitative analysis, we performed a Transwell assay (Fig. 3B). Clones #1 and #6 as well as the HeLa<sup>JLP-shRNA1</sup> cells migrated 30% as efficiently as the HeLa<sup>ctrl</sup> cells (Fig. 3B, *P* < 0.01). We also measured the cell proliferation rates of



**Fig. 2. Immunostaining analysis of the wild-type and JLP KD HeLa cells.** The control (ctrl) and JLP KD cells of clone #6 were cultured on collagen-coated dishes, then fixed and immunostained for paxillin (A) and JLP (B). Scale bars, 20  $\mu$ m. Representative photomicrographs are shown.

these JLP KD cells, and found no obvious difference among the HeLa<sup>ctrl</sup> cells, the HeLa<sup>JLP-shRNA1</sup> cells and clones #1 and #6 (data not shown).

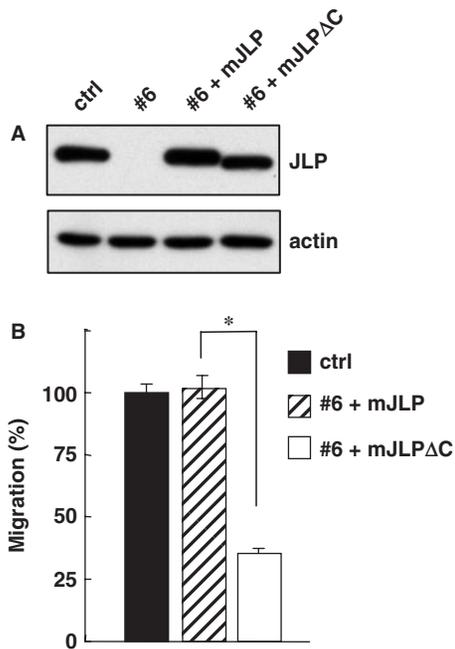
To confirm that the impaired cell migration was due to the depletion of JLP, we re-expressed the wild-type full-length mJLP or its deletion mutant mJLP $\Delta$ C in clone #6. The nucleotide sequence of mJLP cDNA corresponding to JLP-shRNA1 is different from that of human JLP cDNA at 3 out of 21 nucleotides, and thus mJLP mRNA could be resistant to JLP-shRNA1. Indeed, we were able to detect the expression of mJLP in clone #6, and the protein levels of the stably expressing exogenous full-length mJLP and mJLP $\Delta$ C were similar to that of the endogenous JLP in HeLa<sup>ctrl</sup> cells (Fig. 4A). As shown in Fig. 4B, cell migration was rescued by the full-length



**Fig. 3. Impairment of cell migration in JLP KD HeLa cells.** (A) The control (ctrl) HeLa cells, the pooled JLP KD HeLa cells and clones #1 and #6 stably expressing JLP-shRNA1 were replated onto collagen-coated dishes at a saturation density. After being scratched, the wounded monolayers were allowed to heal for 36 h. Scale bar, 200  $\mu$ m. Experiments were performed independently three times, and representative results are shown. (B) The cell migration activity of these cell lines was quantified by a Transwell assay. Values represent the number of migrated pooled KD, clone #1 and clone #6 cells as a percentage of the number of control cells (means  $\pm$  SD from three independent experiments, \* $P$  < 0.01, Student's *t*-test).

mJLP, but not by mJLP $\Delta$ C, indicating that the impaired migration was indeed due to the depletion of JLP, and furthermore suggesting that the C-terminal region of JLP is involved in the regulation of cell migration.

**Involvement of Rac1 and G $\alpha$ <sub>13</sub> in JLP-mediated Cell Migration**—To investigate the molecular mechanism underlying the JLP-mediated cell migration, we examined the involvement of Rac1 and G $\alpha$ <sub>13</sub>, the latter of which has been shown to interact with the C-terminal domain of JLP (11). Consistent with the previous



**Fig. 4. Requirement for JLP in cell migration.** (A) Expression plasmids for the full-length mJLP or mJLP $\Delta$ C were transfected into clone #6, and stable transfectants (#6 + mJLP; #6 + mJLP $\Delta$ C) were selected in the presence of blasticidin S. Total cell lysates were prepared, and subjected to immunoblotting with an anti-JLP antibody. Control (ctrl) and clone #6 cells were used as positive and negative controls, respectively. Actin was used as a loading control. Representative results are shown. (B) Cell migration activity was quantified by a Transwell assay. Values represent the number of migrated stable transfectants as a percentage of the number of control cells (means  $\pm$  SD from three independent experiments, \* $P$  < 0.01, Student's  $t$ -test).

observation by Kashef *et al.* (11), the deletion mutant mJLP $\Delta$ C, but not the full-length wild-type mJLP, failed to bind  $G_{\alpha 13}$  in a transient coexpression experiment in HEK293T cells (Fig. 5A).

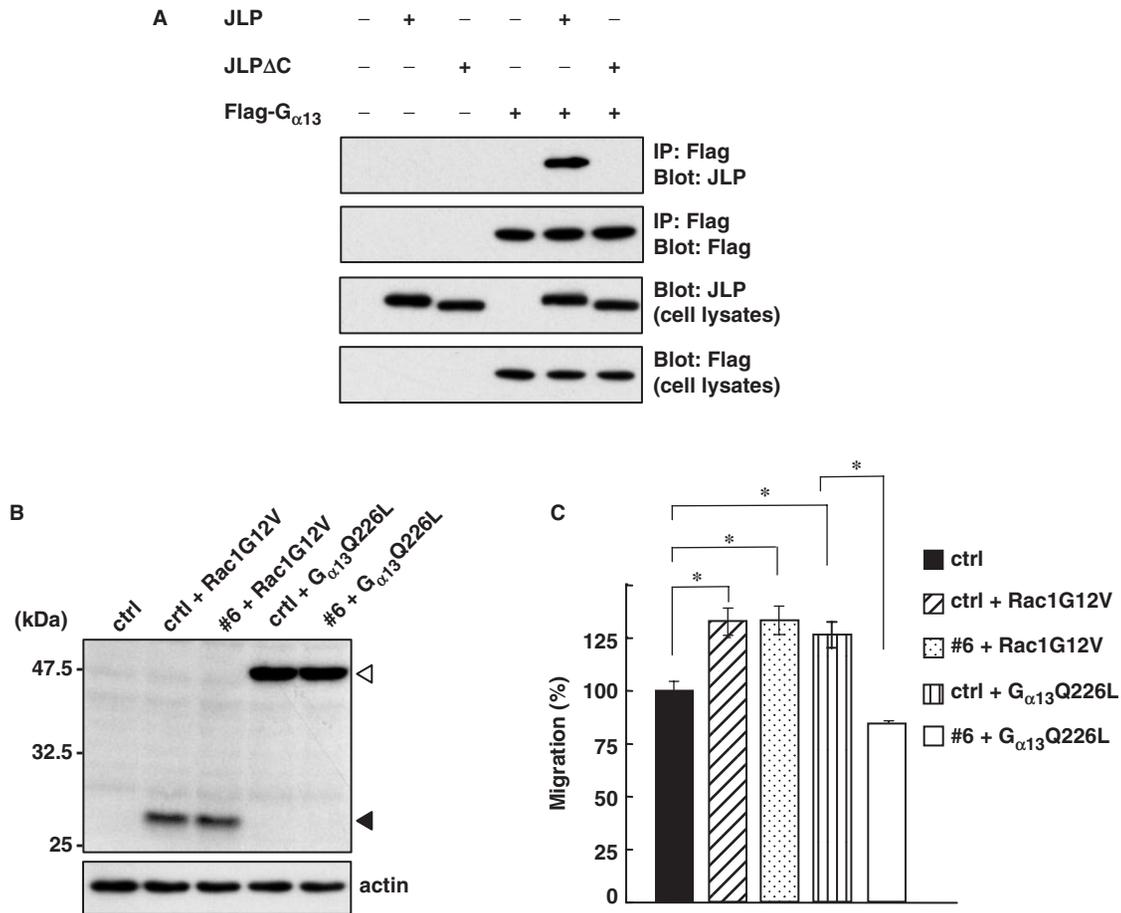
We next introduced expression plasmids for constitutively active Rac1 (Rac1G12V) or  $G_{\alpha 13}$  ( $G_{\alpha 13}$ Q226L) mutants into the HeLa<sup>ctrl</sup> cells and clone #6, selected the cells in the presence of blasticidin S, and analysed the cell migration using a Transwell assay. The protein levels of Rac1G12V stably expressed in the HeLa<sup>ctrl</sup> cells and clone #6 were similar, as was the level of  $G_{\alpha 13}$ Q226L (Fig. 5B). The expression of Rac1G12V or  $G_{\alpha 13}$ Q226L resulted in a similar, significant enhancement of cell migration in the HeLa<sup>ctrl</sup> cells (Fig. 5C). In contrast, Rac1G12V and  $G_{\alpha 13}$ Q226L showed distinct effects on cell migration in clone #6. Rac1G12V almost completely rescued the defect in cell migration; whereas,  $G_{\alpha 13}$ Q226L was unable to fully compensate for the defect in these cells (Fig. 5C). Accordingly, clone #6 expressing  $G_{\alpha 13}$ Q226L showed a significant defect in cell migration compared with the HeLa<sup>ctrl</sup> cells expressing  $G_{\alpha 13}$ Q226L.

Considering the following three lines of evidence, our results strongly suggest that JLP regulates cell

migration through an interaction with  $G_{\alpha 13}$ : (i) JLP KD in HeLa and DLD-1 cells led to a defect in cell migration; (ii) re-expression of the full-length wild-type JLP almost completely rescued the impaired cell migration of the JLP KD HeLa cells; whereas, the re-expression of JLP $\Delta$ C, which failed to bind  $G_{\alpha 13}$ , had little or no effect on the defect in cell migration and (iii) expression of a constitutively active  $G_{\alpha 13}$  enhanced the migration of the control HeLa cells, and the  $G_{\alpha 13}$ -induced cell migration was significantly suppressed in the JLP KD HeLa cells. On the other hand, a constitutively active Rac1 fully compensated for the defective cell migration regardless of the presence or absence of JLP protein, suggesting that Rac1 acts downstream of JLP- $G_{\alpha 13}$  signalling, in which  $G_{\alpha 13}$  activates Rac1 through the relevant guanine exchange factors (18).

**Altered Subcellular Distribution of Active p38 in JLP KD Cells**—Because JLP has been identified as a scaffolding protein for JNK and p38 signalling (6), we analysed the JNK and p38 in HeLa<sup>ctrl</sup> cells and clone #6 by immunoblotting and immunofluorescent staining using antibodies against the phosphorylated, activated forms of JNK (p-JNK) and p38 (p-p38). However, in the absence of extracellular stimuli, the expression of p-JNK and p-p38 was below detection level in both the HeLa<sup>ctrl</sup> cells and clone #6 (data not shown). We therefore examined whether the depletion of JLP perturbed the normal activation and subcellular distribution of JNK and p38 in response to stimuli. The expression levels of p-JNK and p-p38 increased markedly after UV-C irradiation and anisomycin treatment, and no obvious difference in the expression level of p-JNK or p-p38 was observed between the HeLa<sup>ctrl</sup> cells and clone #6 (Fig. 6A; data not shown). In contrast, the subcellular distribution of p-p38 was different between UV-C-irradiated clone #6 and HeLa<sup>ctrl</sup> cells (Fig. 6B and C). After UV-C irradiation, immunofluorescent signals for p-p38 were detected in both the nucleus and cytoplasm of the HeLa<sup>ctrl</sup> cells, whereas almost all of the signal was in the nucleus of clone #6 cells, and there was little or no immunolabeling in the cytoplasm (Fig. 6B, a and b). There were no obvious differences in the immunosignals for p-p38 between the HeLa<sup>ctrl</sup> cells and clone #6 after anisomycin treatment (Fig. 6B, c and d) or for p-JNK in the control and JLP-depleted cells after UV-C irradiation and anisomycin treatment (data not shown). We also examined the subcellular distribution of p-p38 by immunoblotting analysis, using cytoplasmic and nuclear extracts prepared from the HeLa<sup>ctrl</sup> cells and clone #6 (Fig. 6C). The results were consistent with the data obtained by immunocytochemical staining. That is, after UV-C irradiation, p-p38 was detected in both the cytoplasmic and nuclear fractions of the HeLa<sup>ctrl</sup> cells, but it was only observed in the nuclear fraction of clone #6 (Fig. 6C, lanes 2, 5, 8 and 11).

These immunocytochemical and immunoblotting results strongly suggest that the scaffolding protein JLP is involved, at least in part, in the regulation of the subcellular distribution of active p38. Because MAPKs, including p38, translocate upon activation from the cytosol into the nucleus in many different types of cells, JLP may suppress the nuclear



**Fig. 5. Involvement of Rac1 and G $\alpha$ <sub>13</sub> in JLP-mediated cell migration.** (A) Flag- G $\alpha$ <sub>13</sub> was transiently coexpressed with or without the full-length mJLP or mJLP $\Delta$ C in HEK293T cells as indicated. As negative controls, the full-length mJLP or mJLP $\Delta$ C were transiently expressed without Flag- G $\alpha$ <sub>13</sub> in HEK293T cells. Forty-eight hours after transfection, Flag- G $\alpha$ <sub>13</sub> was immunoprecipitated (IP) with an anti-Flag antibody, and the immunoprecipitates were examined by immunoblotting using antibodies against JLP and Flag (upper two panels). Expression of Flag- G $\alpha$ <sub>13</sub>, full-length mJLP, and mJLP $\Delta$ C in the respective cells was examined by immunoblotting (lower two panels). Experiments were performed independently three times, and representative results are shown. (B) Expression plasmids for Flag-Rac1G12V

and - G $\alpha$ <sub>13</sub>Q226L were introduced into the control (ctrl) HeLa cells or clone #6. Stable transfectants (ctrl + Rac1G12V; #6 + Rac1G12V; ctrl + G $\alpha$ <sub>13</sub>Q226L; #6 + G $\alpha$ <sub>13</sub>Q226L) were selected with blasticidin S. Total cell lysates were prepared, and subjected to immunoblotting with an anti-Flag antibody. The white and closed arrowheads indicate the positions of Flag- G $\alpha$ <sub>13</sub>Q226L and -Rac1G12V, respectively. The positions of protein size markers are indicated on the left. Actin was used as a loading control. (C) Cell migration activity was quantified by a Transwell assay. Values represent the number of migrated stable transfectants as a percentage of the number of control cells (means  $\pm$  SD from three independent experiments, \* $P$  < 0.01, Student's  $t$ -test).

translocation of active p38 and contribute to retaining a portion of active p38 in the cytoplasm.

#### ACKNOWLEDGEMENTS

We thank Dr W. C. Hahn (Dana-Farber Cancer Research Institute, Harvard Medical School) for providing the plasmid vectors, pMKO.1-puro, pBabe-puro and pWZL-blast.

#### FUNDING

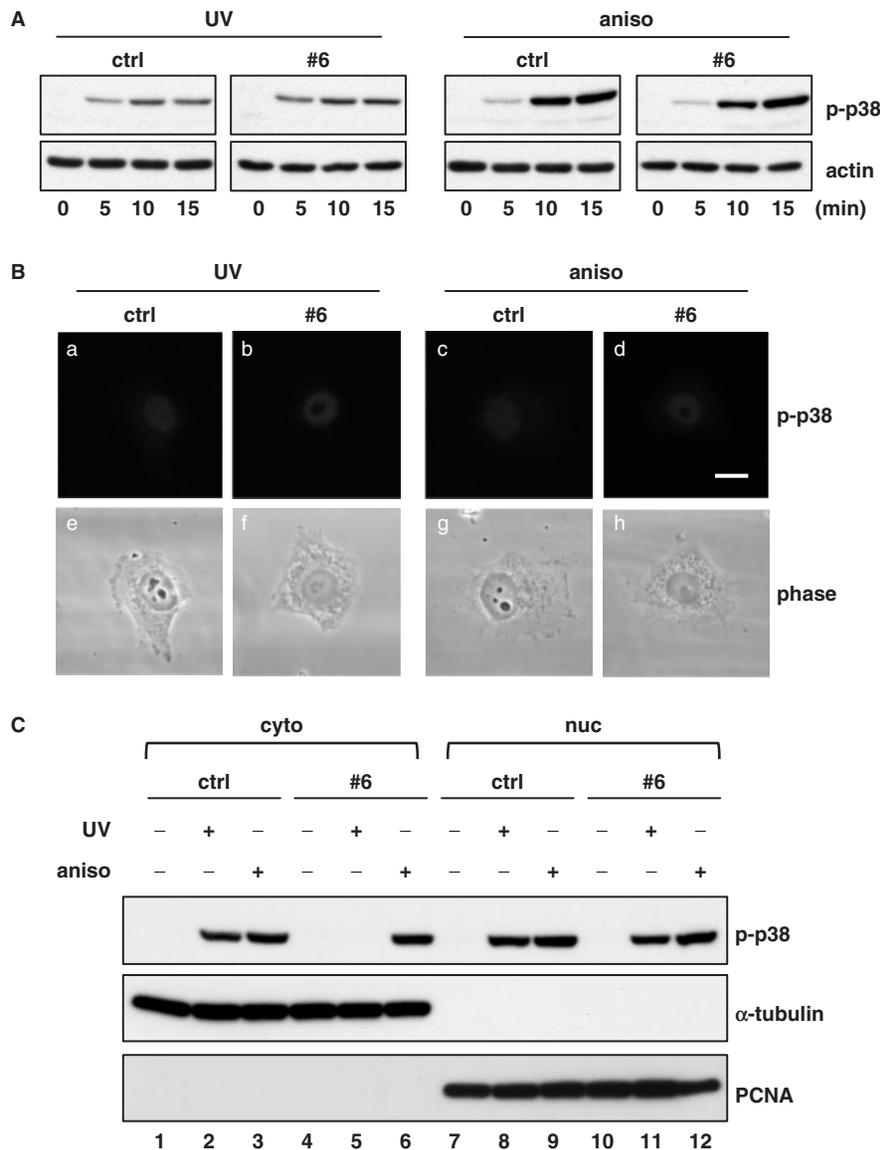
Ministry of Culture, Sports, Science and Technology of Japan.

#### CONFLICT OF INTEREST

None declared.

#### REFERENCES

1. Kyriakis, J.K. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **81**, 807–869
2. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signaling cascades. *Nature* **410**, 37–40
3. Morrison, D.K. and Davis, R.J. (2003) Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annu. Rev. Cell Dev. Biol.* **19**, 91–118
4. Yoshioka, K. (2004) Scaffold proteins in mammalian MAP kinase cascades. *J. Biochem.* **135**, 657–661
5. Dhanasekaran, D.N., Kashef, K., Lee, C.M., Xu, H., and Reddy, E.P. (2007) Scaffold proteins of MAP-kinase modules. *Oncogene* **26**, 3185–3202
6. Lee, C.M., Onésime, D., Reddy, C.D., Dhanasekaran, N., and Reddy, E.P. (2002) JLP: a scaffold protein that tethers



**Fig. 6. Altered subcellular distribution of active p38.** (A) Activation of p38 in response to stimuli in the control (ctrl) HeLa cells and clone #6. Cells were stimulated with UV-C irradiation (UV) or anisomycin (aniso) for the indicated time periods, and the total cell lysates (20 µg/lane) were analysed by immunoblotting with an antibody against p-p38. Actin was used as a loading control. (B) After stimulation of the ctrl HeLa cells and clone #6 with UV or aniso for 15 min, the cells were fixed and immunostained for p-p38 (a–d). Phase-contrast photomicrographs corresponding to the respective fluorescent micrographs are

shown in (e–h). Scale bar, 20 µm. (C) The ctrl HeLa cells and clone #6 were unstimulated or stimulated with UV or aniso for 15 min, and equal amounts (20 µg/lane) of cytoplasmic (cyto; lanes 1–6) and nuclear (nuc; lanes 7–12) fractions prepared from the cells were subjected to immunoblotting with an antibody against p-p38. The relative purity of the cytoplasmic and nuclear fractions was confirmed with the cytoplasmic marker  $\Delta$ -tubulin and the nuclear marker PCNA. Experiments were performed independently three times, and representative results are shown.

JNK/p38MAPK signaling modules and transcription factors. *Proc. Natl Acad. Sci. USA* **99**, 14189–14194

7. Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K. (1999) JSAP1, a novel Jun N-terminal protein kinase (JNK) that functions as a scaffold factor in the JNK signaling pathway. *Mol. Cell Biol.* **19**, 7539–7548
8. Ito, M., Akechi, M., Hirose, R., Ichimura, M., Takamatsu, N., Xu, P., Nakabeppu, Y., Shiba, T., Yamamoto, K., and Yoshioka, K. (2000) Isoforms of JSAP1

scaffold protein generated through alternative splicing. *Gene* **255**, 229–234

9. Kelkar, N., Gupta, S., Dickens, M., and Davis, R.J. (2000) Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. *Mol. Cell Biol.* **20**, 1030–1043
10. Nguyen, Q., Lee, C.M., Le, A., and Reddy, E.P. (2005) JLP associates with kinesin light chain 1 through a novel leucine zipper-like domain. *J. Biol. Chem.* **280**, 30185–30191
11. Kashef, K., Lee, C.M., Ha, J.H., Reddy, E.P., and Dhanasekaran, D.N. (2005) JNK-interacting leucine zipper

- protein is a novel scaffolding protein in the Galpha13 signaling pathway. *Biochemistry* **44**, 14090–14096
12. Takaesu, G., Kang, J.S., Bae, G.U., Yi, M.J., Lee, C.M., Reddy, E.P., and Krauss, R.S. (2006) Activation of p38 $\Delta/\Delta$  MAPK in myogenesis via binding of the scaffold protein JLP to the cell surface protein Cdo. *J. Cell Biol.* **175**, 383–388
  13. Masutomi, K., Yu, E.Y., Khurts, S., Ben-Porath, I., Currier, J.L., Metz, G.B., Brooks, M.W., Kaneko, S., Murakami, S., DeCaprio, J.A., Weinberg, R.A., Stewart, S.A., and Hahn, W.C. (2003) Telomerase maintains telomere structure in normal human cells. *Cell* **114**, 241–253
  14. Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999) Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464–468
  15. Wei, W., Jobling, W.A., Chen, W., Hahn, W.C., and Sedivy, J.M. (2003) Abolition of cyclin-dependent kinase inhibitor p16Ink4a and p21Cip1/Waf1 functions permits Ras-induced anchorage-independent growth in telomerase-immortalized human fibroblasts. *Mol. Cell Biol.* **23**, 2859–2870
  16. Bayarsaikhan, M., Takino, T., Gantulga, D., Sato, H., Ito, T., and Yoshioka, K. (2007) Regulation of N-cadherin-based cell-cell interaction by JSAP1 scaffold in PC12h cells. *Biochem. Biophys. Res. Commun.* **353**, 357–362
  17. Sato, S., Ito, M., Ito, T., and Yoshioka, K. (2004) Scaffold protein JSAP1 is transported to growth cones of neurites independent of JNK signaling pathways in PC12h cells. *Gene* **329**, 51–60
  18. Kelly, P., Casey, P.J., and Meigs, T.E. (2007) Biologic functions of the G12 subfamily of heterotrimeric G proteins: growth, migration, and metastasis. *Biochemistry* **46**, 6677–6687