J. Biochem. **143,** 765–772 (2008)
doi:10.1093/jb/mvn027

Specific Regulation of Cytokine-Dependent p38 MAP Kinase Activation by p62/SQSTM1

Kayoko Kawai[1,2], Akiko Saito[1], Tatsuhiko Sudo[1,*] and Hiroyuki Osada[1,2]

[1]Antibiotics Laboratory and Bioarchitect Research Group, DRI, RIKEN, 2-1 Hirosawa, Wako, Saitama, 351-0198; and [2]Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Saitama, Saitama, 338-8570, Japan

Received December 5, 2007; accepted February 16, 2008; published online February 22, 2008

We have previously shown that p62/SQSTM1 binds to p38. In this study, we identified two association domains of p62 to p38 by conducting co-immunoprecipitation experiments. One domain comprises the amino acids 173–182, named N-terminal p38 interaction (NPI) domain, and the other domain comprises the amino acids 335–344, named C-terminal p38 interaction (CPI) domain. An aspartic acid tripeptide located at 335–337 was required for their association. However, the direct interaction was only observed between the recombinant p38 and the peptide of the NPI domain, but not that of the CPI domain in the surface plasmon resonance analyses. These results suggest that the CPI domain may serve to form a certain conformation suitable for the association with p38. Furthermore, we showed that knockdown of p62 expression by siRNA led to impaired p38 phosphorylation only when HeLa cells were stimulated by cytokine. The critical role of p62 in cytokine-dependent p38 signalling pathway was further confirmed by measuring IL-8 mRNA. Cytokine mRNA is often stabilized *via* p38 pathway. In the absence of p62, IL-8 mRNA induced by IL-1β became more fragile. These data show that p62 specifically regulates cytokine-dependent p38 signalling pathway.

Key words: cytokine, p38, p62, Paget's disease of bone, safety catch.

Abbreviations: IL, interleukin; NF-κB, nuclear factor κB; p38, p38 mitogen-activated protein kinase; siRNA, small interfering RNA; SPR, surface plasmon resonance; SQSTM1, sequestosome1; TNF, tumour necrosis factor.

p62 has been initially identified as a phosphotyrosine-independent ligand for $p56^{lck}$ SH2 domain (*1*). This 440-amino acids protein has an amino-terminal Phox and Bem1p (PB1) domain followed by ZZ type zinc finger domain, TRAF6-binding domain, two PEST sequences and carboxy-terminal ubiquitin-associated (UBA) domain (*2–5*). PB1 domain mediates polymerization of p62 and binding to other PB1 domain containing proteins (*3*, *6*); and PB1 domain and UBA domain contribute to form cytoplasmic inclusion bodies, 'sequestosome', that contain p62 and ubiquitinated proteins (*6*, *7*). This structure may cause proteasomal or autophagosomal degradation (*6*, *8*). Another major role of p62 is described in cytokine receptor signal transduction pathways. The pathway is triggered by the ligand binding to the cognate receptor complex composed of hetero-multimer proteins, such as a receptor interacting protein (RIP) and tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (*3*). RIP interacts with the ZZ domain of p62 and TRAF6 interacts with TRAF6-binding domain of p62. Moreover, p62 interacts with atypical protein kinase C (aPKC) and regulates nuclear factor κB (NF-κB) activation. The knockdown of p62 by anti-sense plasmid severely abrogates NF-κB activation in response to TNF-α and IL-1β. Both NF-κB activation and osteoclastogenesis stimulated by RANK-L (RANK-ligand)/RANK pathway were also impaired in p62 knock out mice (*9*). It seems that p62 may play important roles for the complex formation and modulation of the components in the intra-cellular signalling pathway. Although various mutations in *p62* gene, especially in UBA domain were responsible for 5q35 (PDB3)-linked Paget's disease of bone (PDB) (*10*), the exact function of p62 in bone metabolism remains to be elucidated.

On the other hand, p38 mitogen-activated protein kinase (MAPK) was identified as either an anti-inflammatory drug-binding protein, a lipopolysaccharide activated protein kinase, or a stress-responsive protein kinase (*11*). p38 is implicated as a mediator to transmit intra-cellular signalling for cell survival, differentiation and response to stress including various cytokines *in vitro* and *in vivo*. p38 is dual phosphorylated and activated by its specific upstream kinase MAPK kinase 3 (MKK3) or MKK6. Then p38 activates downstream kinases such as MAPKAPK2/3 (MK2/3) and transcription factors such as ATF2, MEF2C and CHOP through direct phosphorylation. Since MK2 phosphorylates tristetraprolin (TTP), which is a member of a RNA-binding protein (*12*) and regulates stability of mRNAs containing adenine/uridine-rich element (ARE) in their 3′-untranslated regions (3′-UTR), such as IL-8, cyclooxygenase 2 (Cox-2) and TNF-α (*13–15*), p38/MK2 pathway also regulates gene expression post-transcriptionally (*16*). Furthermore, loss of p38 destabilizes MK2 protein

*To whom correspondence should be addressed. Tel: +81 48 467 9542, Fax: +81 48 462 4669, E-mail: sudo@riken.jp

indicating that p38 not only phosphorylates but also stabilizes MK2 protein and MK2 mutually stabilizes p38 (*17*, *18*). However, the precise molecular mechanism how p38 can exert various responses to different stimuli are unclear. We previously showed that p62 binds and regulates p38 *in vitro* (*19*). The study dropped a hint to explain how p38 can be regulated in various environmental stimuli and in different signal transduction pathways using extra factor(s).

Here, we report that p62 associates with p38 using two domains which are different from the protein interaction domains already reported. Furthermore, we demonstrate that one of these domains binds to p38 directly, while the other domain does not bind to p38 directly and may serve to form a certain conformation suitable for the association with p38.

We also report that the loss of the p62 specifically reduces the cytokine-dependent activation of p38. Thus, p62 appears to function as a stimulation-specific regulator to p38.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human TNF-α was purchased from R&D Systems. Recombinant human Interleukin-Iβ was from Pepro Tech EC LTD. Anisomycin was from SIGMA. Actinomycin D was from Calbiochem. The anti-HA antibody (F-7) was from Santa Cruz Biotechnology. Anti-phospho-p38 MAPK (T180/Y182) antibody (3D7), anti-phospho-MAPKAPK-2 (Thr334) and anti-MAPKAPK-2 were from Cell Signaling. Anti-p38 (*20*) and anti-p62 antibody were raised by immunizing rabbits with an affinity purified recombinant glutathione *S*-transferase-mouse p38α (GST-mp38) and GST-p62. The anti-GST-mp38 and p62 sera were fractionated by ammonium sulphate precipitation and repeatedly applied to GST-column to remove antibodies against GST and bacterial proteins. Then, partially purified anti-GST-mp38 serum was multi-step affinity purified by binding to His-human p38 column. Pre-stained protein molecular markers were from Bio-Rad.

Cell Culture—HeLa cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (JRH Biosciences), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma) and 1 mM MEM sodium pyruvate (GIBCO) at 37°C with 5% CO_2.

Plasmid Construction—The plasmids expressing 3HA-tagged p62 and its mutants were constructed as follows. First, p62 fragment including entire coding region was obtained from pBKp62 (*19*) and inserted into pSRα3HA plasmid. C-terminal deletion mutants of p62 and 4A, 3A, 7A, 3N mutants were constructed as described in the previous report (*21*). We designed the primers as follows. 5′-GGTCCAGTCATCTTATCCTCCTGAACAG-3′ for p62-334, 5′-AGACGGGTCCACTTATTTTGAAGACAGATG-3′ for p62-344, 5′-AGCCTCTGGCGGTCAATGTGGGTACAAGGC-3′ for p62-385, 5′-CCGAAGTGTCCGTGTGCCACCGCCGCGAGCCAGGCGCTGTGCGAGAAGCC-3′ for p62-4A, 5′-GGTCAGGGCGCTTGCTGCCCCTCCGTGCTC-3′ for p62-3A, 5′-GAAGACAGATGGGTCCAGTTATTATTTCCTCCTGAACAGTTATC-3′ for p62-3N, 5′-GCCCTCAGACAGGGCCCCGAAGGGGCTGGG-3′ for p62-H174A, 5′-TGCGAGAAGCCCTGAGACAGGTGCCCGAAG-3′ for p62-E177Q, 5′-CCAGCGGCTGTGCGCGAAGCCCTCAGACAG-3′ for p62-S180A, 5′-AGCCAGCGGCTGGCCGAGAAGCCCTCAGAC-3′ for p62-H181A and 5′-CCGGAGCCAGCGGGCGTGCGAGAAGCCCTC-3′ for p62-S182A were synthesized and performed PCR. Next, we constructed p62 N-terminal deletion mutants using pcDNA3 3HA-p38 (*22*) as a backbone. We performed PCR amplification using pSRα3HA-p62 as a template and primers mentioned as follows. 5′-AATCGATGACCAAGCTCGCATTC-3′ for p62-Δ163, 5′-AATCGATGGGGCACCTGTCTGAGG-3′ for p62-Δ172 and 5′-AATCGATGCGCTGGCTCCGGAAGGTG-3′ for p62-Δ182, in combination with 5′-GCAGGGCCCTCACAACGGCG-3′ for common anti-sense primer were used. The PCR products were then cloned into pGEM^{R}-T Easy Vector (Promega) and digested by newly generated *Cla*I and *Apa*I sites. These fragments were inserted into the *Cla*I and *Apa*I sites of pcDNA3 3HA-hp38. The GST-human p38 (hp38) expression plasmid was constructed as follows. A new *Cla*I site was made pGEX-KG (*23*), and the plasmid named pGEX-CL. *Cla*I and *Sac*I fragments from pcDNA3 3HA-hp38 (*22*) were inserted into the same site of pGEX-CL. The DNA sequences were confirmed by the ABI system.

Co-Immunoprecipitation—Into each well of a 6-well plate 1.0×10^5 cells were seeded. The next day, 3HA-p62 or mutant expression vector was transfected with Effectene^{R} (Qiagen) according to the manufacturer's instructions. These cells were grown in DMEM supplemented with 10% FCS for 24 h. Co-immunoprecipitation assays were conducted using polyclonal anti-p38 antibody. Cells were rinsed with PBS supplemented with 0.5% sodium orthovanadate, and lysed in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenylphosphate, 1 mM PMSF and 10 μg/ml leupeptin. Then, the samples were further lysed by sonication followed by 15-min centrifugation at 15,000 r.p.m. at 4°C. These lysates were precleared by the addition of protein A Sepharose (GE Healthcare) followed by brief centrifugation. Then, antibody pre-bound protein A Sepharoses were mixed with the lysates and rotated for 3 h at 4°C. After five washes with the lysis buffer, proteins that bound to the affinity beads were separated by 7 or 10% SDS–PAGE followed by immunoblotting.

RNA Interference—Small interfering RNA (siRNA) for silencing the expression of p62 (sense: 5′-UACAAAUUUAACAGGAUGGdTdT-3′, anti-sense: 5′-CCAUCCUGUUAAAUUUGUAdTdT-3′) and the control siRNA against luciferase GL2 (sense: 5′-CGUACGCGGAAUACUUCGAdTdT-3′, anti-sense: 5′-UCGAAGUAUUCCGCGUACGdTdA-3′) were synthesized by Nippon EGT. Into each well of a 12-well plate 0.25×10^5 cells were seeded. The next day, cells were transfected with 50 pmol/well of siRNA using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. Six hours after transfection, medium was changed. Forty-eight hours after transfection, cells were stimulated with 100 ng/ml anisomycin,

0.5 M sorbitol, 0.5 M NaCl, 100 ng/ml TNF-α, 100 ng/ml IL-1β or 200 J/m^2 UV irradiation. After 15 min, the whole-cell lysates were prepared by the addition of SDS sample buffer and were separated by 12% SDS–PAGE followed by immunoblotting.

RT–PCR Analysis—We performed RT–PCR analysis to examine IL-8 mRNA expression and stability in p62 knockdown cells. Forty-eight hours after transfection with siRNA, cells were treated with 20 ng/ml IL-1β. Two hours after IL-1β stimulation, cells were treated with 4 μM actinomycin D for inhibiting new RNA synthesis. Total RNA was prepared and cDNA was synthesized as mentioned previously (*22*). We designed primers 5′-AAGC TGGCCGTGGCTCTCTTG-3′corresponding to 10–30 of the IL-8 nucleotides, and 5′-GAATTCTCAGCCCTCTTC-3′ corresponding to 296–279 to obtain a 287-bp PCR product. GAPDH was used as a control. We also designed primers 5′-GAAGGTGAAGGTCGGAGTC-3′ corresponding to the nucleotide between 6 and 24, and 5′-TCTCC ATGGTGGTGAAGAC-3′ corresponding to the nucleotide between 319 and 301 to obtain a 314-bp PCR product.

Preparation of Recombinant Protein—The GST-hp38 expression plasmid was expressed in a bacterial strain, BL21(DE3), and grown in LB media (100 μg/ml ampicillin) at 37°C until the cell density reached an A_{600} of 0.8. The culture was induced with 1 mM IPTG and incubated at 37°C for 2 h. The harvested bacterial cells were re-suspended in lysis buffer (50 mM Tris, pH 7.5, 25% sucrose) with 0.2 mg/ml Lysozyme and 0.01 mM $MgCl_2$ added. On ice for 1 h, flash-frozen cells were thawed twice, and lysed by adding 1% NP-40 followed by gentle sonication. After centrifugation at 10,000 r.p.m. for 15 min at 4°C, the supernatant was mixed with 1 ml 50% glutathione–agarose beads (SIGMA) and rotated at 4°C. After 2 h, the beads were poured into a column and washed with 20 ml wash buffer (20 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT). Fusion protein was eluted with elution buffer (5 mM GSH, 50 mM Tris, pH 9.6) and dialysed into PBS with 10% glycerol.

Peptides—Peptides were synthesized by Research Resources Center/Brain Science Institute/RIKEN. We designed the peptides as follows. p62-178 (corresponding to 178–190 of p62:GFSHSRWLRKVKH). p62-262 (corresponding to 262–274 of p62:GGKRSRLTPVSPE). p62-330 (corresponding to 330–342 of p62:NCSGGDDDWTHLS). The peptides corresponding to the p38-docking domain of MEF2A (*24*) that is a specific substrate for p38. MEF2A(WT):(corresponding to 266–281 of MEF2A: MNSRKPDLRVVIPPSS). MEF2A(M1) (MEF2A mutant 1:MNS**AA**PDLRVVIPPSS). MEF2A(M2) (MEF2A mutant 2:MNSRKPD**ARA**VIPPSS). Residues altered in the mutants of MEF2A are shown in bold.

SPR Analysis of p38-Peptide Interaction on Photo-Cross-Linker-Coated Gold Substrate (PGS)—The peptides were immobilized on PGSs as described (*25*) with an improved linker. DMSO solution (10 mM) of each peptide was spotted on PGSs. After they were dried *in vacuo*, the substrates were exposed to a dose of irradiation of 2.8 J/cm^2 at 365 nm, and then were washed with water for 12 h. The array was placed into SPR imaging instrument, and incubated with 0.1% BSA blocking solution (10 mM HEPES, 150 mM NaCl, pH 7.4) for 5 min. After washing with HEPES buffer, GST-hp38 (0.3 mg/ml, 10 mM HEPES, 150 mM NaCl, pH 7.4) was injected to the array surface at 0.1 ml/min and incubated for 10 min. All SPR experiments were performed at 30°C. The SPR image and signal data were collected with an SPR analysis program (TOYOBO) (*26*).

RESULTS AND DISCUSSION

Association Domains of p62 to p38—Overall protein structure of p62 and protein interaction domains identified so far are shown in Fig. 1A. Since p62 proteins produced in bacteria were barely soluble, we applied co-immunoprecipitation methods to dissect the association domain(s) of p62 to p38. However, C-terminally Flag-tagged p62 (p62-Flag) was used to show the interaction between p38 and p62 in the previous study (*19*); in this study, we first asked if p38 binds to N-terminally 3HA-tagged p62 (3HA-p62). As shown in Fig. 1B, 3HA-p62 can be co-immunoprecipitated by anti-p38 antibody (lane 4) but not by a control rabbit pre-immune serum (lane 3) in transient transfection assay in HeLa cells. This result indicates that their interaction can be analysed by using N-terminally 3HA-tagged p62.

We constructed plasmids expressing amino acid substituted or deletion mutants of 3HA-p62. Then, we transiently transfected these plasmids into HeLa cells, and pulled down p62 variants that were associated with p38 by using an antibody recognizing p38. Since p38 binds to cognate substrates through their positively charged amino acid cluster (*27*), we first searched for such clusters in p62. We found two possible clusters,

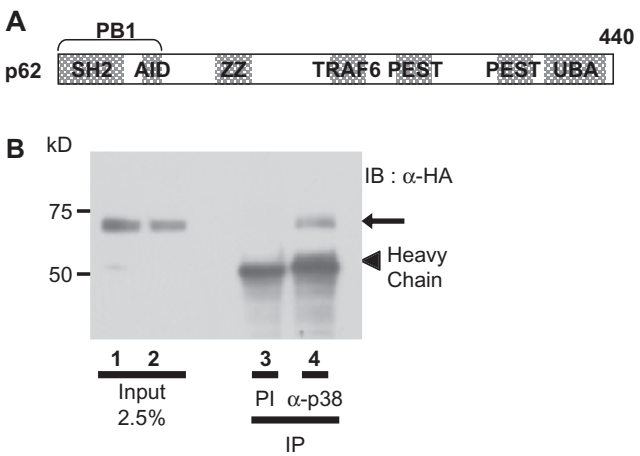

Fig. 1. **p38 binds to p62.** (A) The functional domains of p62. PB1: Phox and Bem1p domain; SH2: Src homology 2 domain; AID: atypical protein kinase C (aPKC) interaction domain; ZZ: zinc finger domain; TRAF6: TRAF6-binding sequence; UBA: ubiquitin-associated domain. (B) 1.0×10^5 cells of HeLa cells were seeded in 6-well plate and cultured for 24 h. Then, 3HA-p62 expression vector was transfected. Twenty-four hours after transfection, cell extracts were immunoprecipitated with an anti-p38 antibody (α-p38) or pre-immune serum (PI), and immunoprecipitates were analysed by 10% SDS–PAGE followed by immunoblotting with an anti-HA antibody. An aliquot of 2.5% input of pre-cleared cell extracts were also shown (lanes 1 and 2). arrow: 3HA-p62.

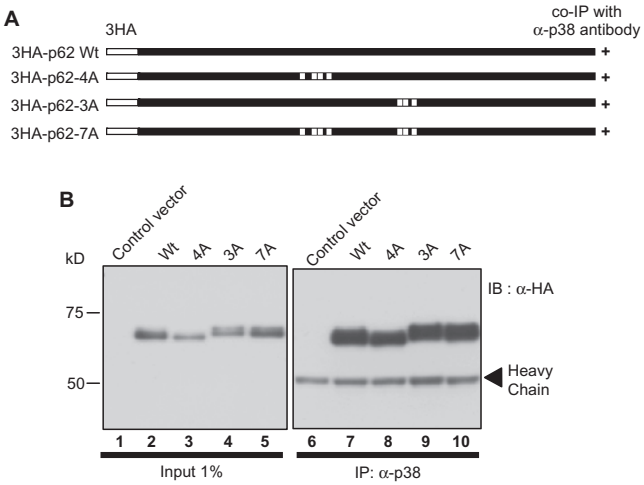

Fig. 2. **Basic amino acid clusters of p62 are not important for the association with p38.** (A) The structures of p62 point mutants. R183, R186, K187 and K189 were mutated to alanine in p62-4A mutant; K264, R265 and R267 in p62-3A; all seven mutations in p62-7A. The results of co-immunoprecipitation (co-IP) analyses with anti-p38 antibody are summarized on the right. (B) 1.0×10^5 cells of HeLa cells were seeded in 6-well plate and after 24 h, 3HA-p62 or mutant expression vector was transfected. Twenty-four hours after transfection, cell extracts were prepared and immunoprecipitated with the anti-p38 antibody (α-p38). Then, immunoprecipitates were analysed by 10% SDS–PAGE followed by immunoblotting with an anti-HA antibody. An aliquot of 1% input of pre-cleared cell extracts were also shown (lanes1–5).

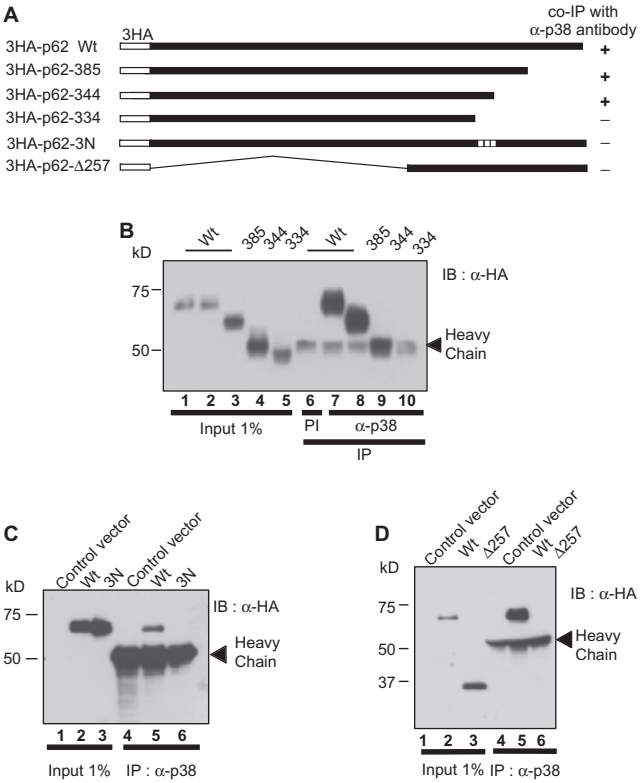

Fig. 3. **Analysis of the p62 domains required for the association with p38.** (A) The structures of p62 mutants. Aspartic acid 335, 336 and 337 were mutated to asparagine in p62-3N. The results of co-IP analyses are summarized on the right. (B–D) 1.0×10^5 cells of HeLa cells were seeded in 6-well plate and cultured for 24 h. Then, 3HA-p62 or mutant expression vector was transfected. Twenty-four hours after transfection, cell extracts were immunoprecipitated with an anti-p38 antibody (α-p38) or pre-immune serum (PI), and immunoprecipitates were analysed by 7% (B) or 10% (C, D) SDS–PAGE followed by immunoblotting with an anti-HA antibody. An aliquot of 1% input of pre-cleared cell extracts were also shown (B: lanes 1–5, C, D: lanes 1–3).

namely arginine (R)183, 186, lysine (K)187, 189 and K264, R265, 267. These basic residues were substituted to alanines (A), p62-4A (R183/186A and K187/189A), p62-3A (K264A and R265/267A) and p62-7A (R183/186/265/267A and K187/189/264A) to analyse their roles (Fig. 2A). As shown in Fig. 2B, these three mutant proteins can be precipitated almost equally to the wild type (lanes 7–10) and comparable amounts of p62 proteins were expressed (lanes 2–5). Results obtained in the experiments were summarized in Fig. 2A (far right) and indicated that p38 interacts with p62 in a different manner from the one with positively charged amino acid cluster.

Next, co-immunoprecipitation assays using C-terminal deletion mutants were conducted. Structures of mutants tested were depicted in Fig. 3A. As shown in Fig. 3B, p62-334 did not co-precipitate with p38 (lane 10) even though the expression of this mutant was comparable to those of other mutants (lanes 1–5). This indicates that amino acids 335–344 of p62 are required for association with p38. Furthermore, we found unique consecutive three aspartic acid residues (D) ranging from 335 to 337 in this region (Fig. 3A). To discover whether the negatively charged amino acid cluster was important for their association, we changed these aspartic acid residues to asparagine residues (N) to neutralize the electric charge (Fig. 3A, p62-3N). The results of co-immunoprecipitation assay clearly showed that aspartic acid to asparagine substitution (p62-3N) almost completely abolish its ability to associate with p38 (Fig. 3C, lane 6). Then, we asked if this domain is sufficient for association of p62 with p38. As shown in Fig. 3D, p62-Δ257, a deletion mutant containing this domain could not be co-precipitated with p38 complex (Fig. 3D, lane 6). Results obtained in the experiments were summarized in Fig. 3A (far right) and it indicates that amino acids 335–344 are essential but not sufficient for their association.

We assumed that there must be additional domain(s) necessary for their association. Therefore, we constructed N-terminal deletion mutants and performed similar co-immunoprecipitation assays. Structures of mutants tested were depicted in Fig. 4A. As shown in Fig. 4B, even though the wild-type and all mutants are equally expressed (lanes 1–4) and p62-Δ172 binds to p38, p62-Δ182 that lacks amino acids 1–182 lost its ability to bind to p38 (compare lanes 7 and 8). Results obtained in the experiments were summarized in Fig. 4A (far right) and it indicates that amino acids 173–182 of p62 are also necessary for interaction with p38. Hereafter, we call the former domain ranging from 335 to 344 as C-terminal

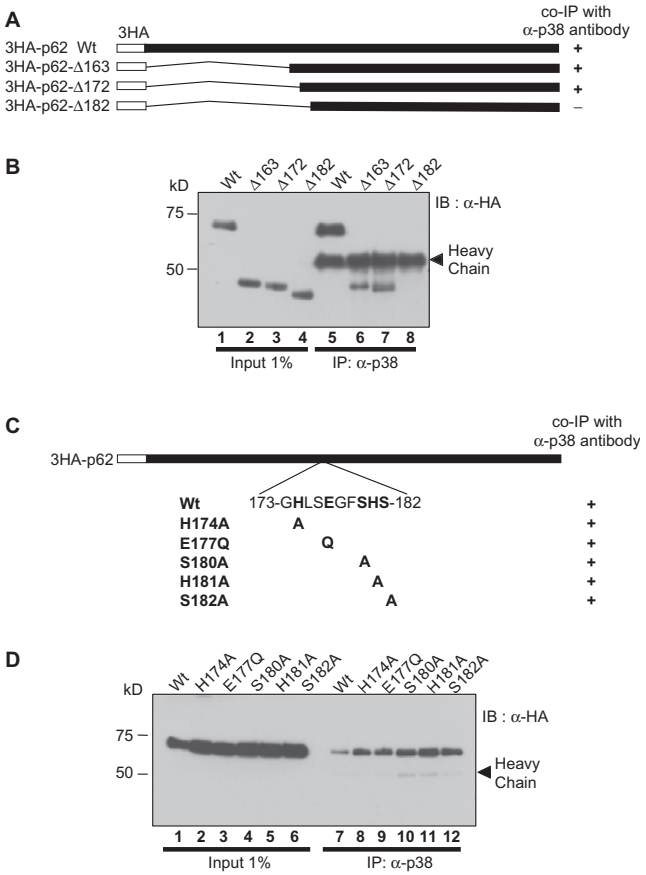

Fig. 4. **The second domain of p62 required for the association with p38.** (A) The structures of p62 N-terminal deletion mutants are shown. The results of co-IP analyses with anti-p38 antibody are summarized on the right. (B, D) 1.0×10^5 cells of HeLa cells were seeded in 6-well plate and after 24 h, 3HA-p62 or mutant expression vector was transfected. Twenty-four hours after transfection, cell extracts were prepared and immunoprecipitated with the anti-p38 antibody (α-p38). Then, immunoprecipitates were analysed by 10% SDS–PAGE followed by immunoblotting with an anti-HA antibody. An aliquot of 1% input of pre-cleared cell extracts were also shown (B: lanes 1–4, D: lanes 1–6). (C) Amino acid sequences of the wild type (Wt) and point mutants of p62 are shown. The results of co-IP analyses with anti-p38 antibody are summarized on the right.

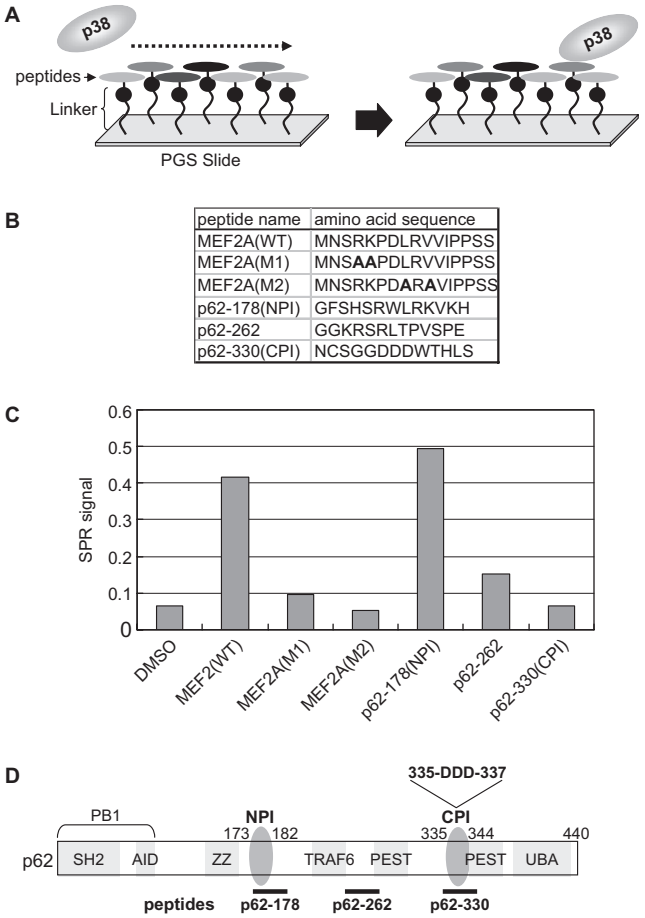

Fig. 5. **The peptide corresponding to NPI domain, but not CPI domain, directly binds to p38.** (A) Overview of surface plasmon resonance (SPR) analysis. The peptides were immobilized on PGSs (photo-cross linker coated gold substrates). We detected interactions between these immobilized peptides and GST-hp38 in solution by SPR analysis. (B) The amino acids sequence of synthesized peptides is shown. Residues altered in the mutant MEF2A peptides, MEF2A(M1) and MEF2A(M2) are shown in bold. (C) SPR signal data were collected with an SPR analysis program (TOYOBO), and the maximum SPR signal strengths of each peptide were shown. (D) The association domains of p62 to p38 newly identified in this study were highlighted. NPI: N terminal p38 interaction domain; CPI: C terminal p38 interaction domain. Relative positions of peptides used in the assay were also shown.

p38 interaction (CPI) domain, and the latter one ranging from 173 to 182 as N-terminal p38 interaction (NPI) domain. To identify the amino acids responsible for their binding, we further tested an amino acid substituted mutants. Five mutants examined were summarized in Fig. 4C. As shown in Fig. 4D, all mutants could bind to p38 (lanes 8–12). Since we could neither identify the amino acid(s) responsible for their binding nor the mode of p62 binding to p38 in this co-immunoprecipitation assay, we next conducted a surface plasmon resonance (SPR) assay by using GST-hp38 and the peptides of p62 to identify the residue(s) responsible for their direct binding.

Overview of SPR analysis is shown in Fig. 5A and the peptide sequences immobilized on PGSs were summarized in Fig. 5B. First, we examined the binding of the GST-hp38 with a peptide named MEF2A(WT) derived from Myocyte enhancer factor 2A (MEF2A) that is a specific substrate of p38, and MEF2A(WT) has been identified as a docking domain peptide for p38 (*24*). We also tested if MEF2A(M1) and MEF2A(M2), that are mutants of MEF2A(WT) and exhibited reduction of the affinity to p38 (*24*), cannot bind to p38 in this analysis. Specific binding was only seen with the MEF2A (WT), but not with the mutants (Fig. 5C) indicating that this experimental system works well to detect the specific binding to p38. Among the peptides derived from p62 (Fig. 5B and C), the peptide corresponding to the NPI domain (p62-178) only showed the binding to GST-hp38 (Fig. 5C). Neither the peptide corresponding to the positively charged amino acid cluster (p62-262) nor CPI domain (p62-330) could bind to GST-hp38 (Fig. 5C).

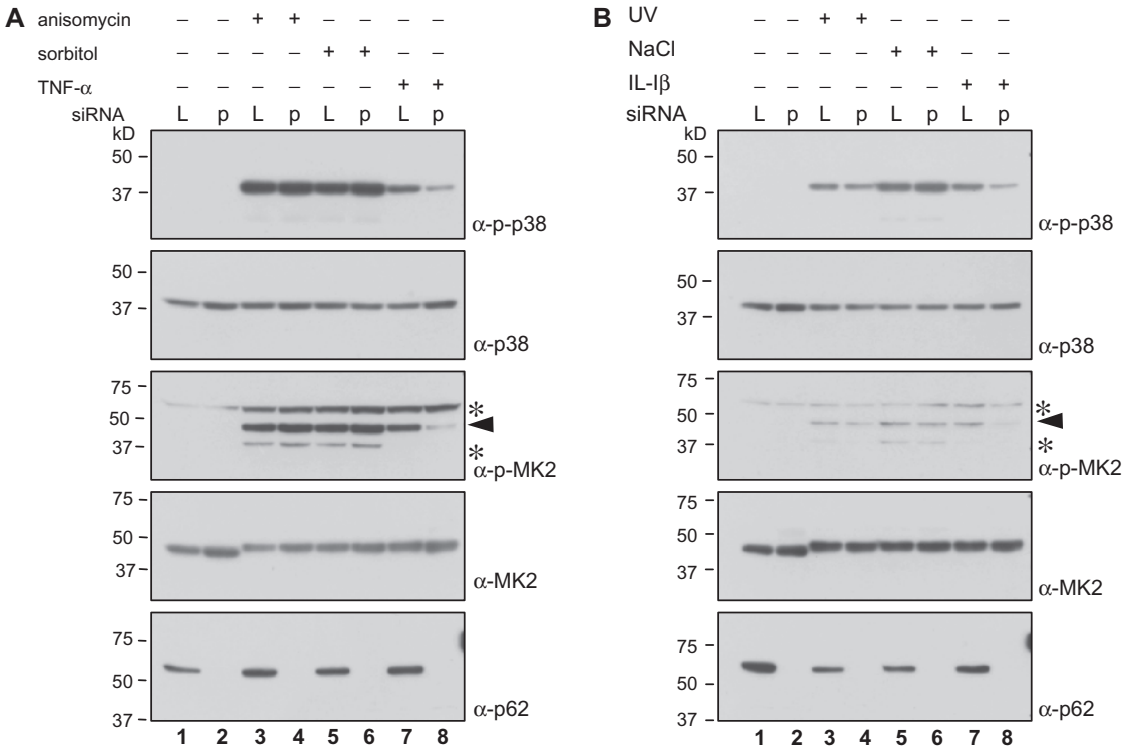

Fig. 6. **Cytokine-dependent p38 activation is regulated by p62.** (A, B) 0.25×10^5 cells of HeLa cells were seeded in the 12-well plate. The cells were cultured for 24 h and transfected with 50 pmol of control (for Luciferase GL2; L) or the p62 (p) siRNA. Forty-eight hours after transfection, cells were treated 100 ng/ml anisomycin (A, lanes 3 and 4), 0.5 M sorbitol (A, lanes 5 and 6), 100 ng/ml TNF-α (A, lanes 7 and 8), 200 J/m^2 UV irradiation (B, lanes 3 and 4), 0.5 M NaCl (B, lanes 5 and 6) or 100 ng/ml Il-1β (B, lanes 7 and 8). After 15 min, cells were harvested with 100 μl of 1 × SDS sample buffer and analysed by 12% SDS–PAGE followed by immunoblotting with anti-phospho-p38 (α-p-p38), anti-p38 (α-p38), anti-phospho-MAPKAPK2 (α-p-MK2), anti-MAPKAPK2 (α-MK2) and anti-p62 (α-p62) antibodies (from top to bottom panel). *:non-specific signal.

These results demonstrated that only the NPI domain of p62, but not CPI domain containing aspartic acid tripeptide, is directly binding to p38. The aspartic acid tripeptide, functionally named 'safety catch', was first described in caspase-3 (*28*). The safety catch was demonstrated to sense intra-cellular acidification to entail protein configuration such as the conformational change of caspase-3 to permit commitment to apoptosis. We speculate that CPI domain of p62 serve to form a certain conformation suitable for the association with p38.

p62 Regulates p38 Phosphorylation by TNF-α and IL-1β—To investigate the functional relevance of their interaction in the regulation of p38 signalling pathway, we tried to override endogenous p62 by expressing mutants in transient transfection experiments. However, since endogenous p62 is too abundant to be overridden by ectopically expressed mutant p62, we changed our strategy to suppress p62 expression by RNA interference using siRNA instead. Transfection of HeLa cells with p62-specific siRNA reduced p62 expression almost completely as observed by western blot analysis (Fig. 6A, bottom panel, lanes 1 and 2). We next asked whether knockdown of p62 expression affects p38 pathway. We compared phosphorylation levels of p38 and MAPKAPK2 (MK2) that is a specific substrate for p38 in HeLa cells with or without p62 by various p38-activating stimuli. When HeLa cells were stimulated by anisomycin, sorbitol (Fig. 6A, lanes 3–6), UV irradiation and NaCl (Fig. 6B, lanes 3–6), the absence of p62 did not clearly affect on the phosphorylation levels of p38 and MK2 (Fig. 6A and B, top and third panels, compare lanes 3 and 4, 5 and 6). However, when HeLa cells were treated with TNF-α, the amounts of phosphorylated p38 and MK2 were clearly decreased in p62 knockdown cells compared with control cells (Fig. 6A, top and third panels, lanes 7 and 8). This is also the case for another cytokine, IL-Iβ (Fig. 6B, top and third panels, lanes 7 and 8). The total amounts of p38 and MK2 were comparable through the experiments (Fig. 6A and B, second and fourth panels). Similar results were also obtained using three different siRNAs to knock down p62 (data not shown). In the cytokine-induced p38 MAP kinase pathway, each cytokine binds to initiate its signal through cognate receptor, such as TNF-α to TNFR1 or IL-1β to IL-1R, respectively. As is already known, p62 is a component of these receptor complexes and knockdown of p62 decreases activation of NF-κB possibly *via* aPKC pathway-dependent manner (*3*). On the other hand, anisomycin, osmotic stress and UV irradiation strongly activate p38, but the mechanism, especially 'receptor or sensor' of these stimuli, is yet unclear. It is the first time to discriminate the molecular mechanism(s) leading to p38 phosphorylation by cytokine *via* p62 and

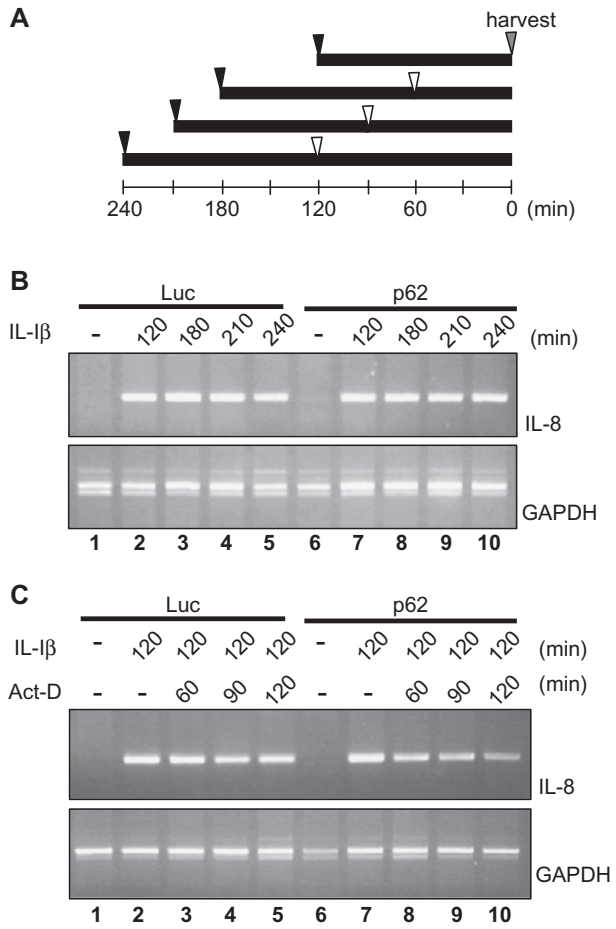

Fig. 7. **IL-1β-induced IL-8 mRNA is destabilized in p62 knockdown cells.** (A) Experimental design was shown. Briefly, 0.5×10^5 cells of HeLa cells were seeded in the 6-well plate. The cells were cultured for 24 h and transfected with 100 pmol of p62 or control (for Luciferase GL2; Luc) siRNA. Forty-eight hours after transfection, cells were treated with 20 ng/ml IL-1β. Solid arrowheads indicate the time points when 20 ng/ml of IL-1β was administrated (for B and C). And white arrowheads indicate the time points when 4 μM actinomycin D was administrated (for C). Cells were harvested and total RNA was prepared and subjected to RT–PCR for IL-8 and GAPDH mRNAs.

by other stimuli. Thus, these results indicate that p62 is an important intermediary not only for NF-κB signalling pathway but also for p38 signalling pathway initiated from specific receptors which form multi-meric complexes including p62.

p62 Plays an Important Role in Cytokine mRNA Stabilization—p38 has been shown to stabilize mRNA containing ARE in 3′UTR, often seen in cytokine mRNAs, through the regulation of a specific downstream substrate, MK2 (*12*, *16*). Since p62 is involved in cytokine-induced activation of p38 and MK2, we asked if the loss of p62 affects mRNA stability. IL-8 mRNA has ARE sequence and is stabilized by cytokine stimulation resulting in active IL-8 production. We analysed IL-1β-dependent IL-8 mRNA stability in HeLa cells by RT–PCR. Experimental designs were summarized in Fig. 7A. As shown in Fig. 7B, IL-8 mRNA was more abundant in response to IL-1β regardless of p62 (lanes 1 and 2, 6 and 7). The amounts of IL-8 mRNA are consistent in this experimental time range in the presence or absence of p62 (Fig. 7B, lanes 2–5 and 7–10). There are two major mechanisms affecting the amount of mRNA, namely transcription and mRNA stability. In general, cytokine strongly activates many transcription factors inducing transcriptions of cytokine genes. To exclude new mRNA synthesis, we treated cells with actinomycin D and found that there is clear decrease of IL-8 mRNA only in p62 knockdown cells (Fig. 7C, lanes 7–10). This result indicates that loss of p62 destabilizes IL-1β-induced IL-8 mRNA. Although it is difficult to completely separate cytokine-induced mRNA stability from transcription, these data indicate that p62, at least in part, plays a role in cytokine-induced mRNA stabilization as well as p38 does.

In this study, we have shown that two domains of p62, amino acid residues of 173–182 (NPI domain) and 335–344 (CPI domain), are required for its interaction with p38 (Figs 3B and D, and 4B). Further investigation using SPR analysis showed that the NPI domain may directly bind with p38 (Fig. 5C). Although an aspartic acid tripeptide in CPI domain of p62 is indispensable for the interaction with p38, this domain may not directly bind with p38. These two domains are different from previously identified domains involved in the interactions between p62 and other signal molecules, namely PB1 domain, ZZ zinc finger domain, TRAF6-binding sequence and UBA domain (Fig. 5D). Therefore, p38 may be able to integrate in the p62 complex without physically competing with the other interactions.

Moreover, we showed that p62 specifically regulates cytokine-dependent p38 MAP kinase activation and loss of p62 leads to deregulation of cytokine-induced mRNA stability possibly *via* p38 pathway corruption. Thus, p62 appears to function as a stimulation-specific regulator to p38. The results obtained in this study may help to explain the complex p38 function in the inflammatory response.

We are grateful to Amy Yee and Eugene Ong for reviewing manuscripts and to Rie Nakazawa for sequencing. This work was supported by 'Bioarchitect Research Project' from RIKEN to T.S.

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