P66^{ShcA} interacts with MAPKAP kinase 2 and regulates its activity

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Abstract Three mitogen activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2, MK2) interacting proteins were identified using a yeast two-hybrid approach. ShcA, a signaling phospho-protein, human polyhomeotic 2 (HPH2), a transcriptional regulator, and highly similar to smoothelin (HSTS), which is related to the cytoskeletal associated protein smoothelin, interact specifically with MK2. The interaction of MK2 with the 66 kDa isoform of ShcA, p66^{ShcA}, and HPH2 was confirmed using co-immunoprecipitation. MK2 is activated with p66^{ShcA} co-expression and p66^{ShcA} is an in vitro substrate for MK2, further demonstrating their association and suggesting a biological role for p66^{Shc} in MK2 activation.

Key words: Mitogen activated protein kinase-activated protein kinase 2; p66^{ShcA}; Human polyhomeotic 2; Highly similar to smoothelin; Heat shock protein 27

1. Introduction

Mitogen activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2 or MK2) is a serine threonine kinase directly phosphorylated and activated by the p38 mitogen activated protein kinase (MAPK)/reactivating kinase [1], the mammalian orthologue of Hog1 which is required for adaptive responses to osmotic stress in *Saccharomyces cerevisiae* [2]. P38 MAPKs are activated by cellular stress including osmotic and heat shock, anisomycin, H_2O_2, and UV light as well as by several pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL1) and by bacterial lipopolysaccharide (LPS). The p38 MAPKs, in turn, regulate a number of environmental stress responses including actin microfilament dynamics, levels of intracellular reactive oxygen species (ROS), apoptosis, and the biosynthesis of several proinflammatory cytokines including TNF-α, IL1, and IL6 [3–5].

MK2 is likely to mediate several p38 regulated pathways since it is a direct substrate for p38. In addition, cells isolated from mice deleted for MK2 are deficient in the LPS induced biosynthesis of several pro-inflammatory cytokines regulated by p38 including TNF-α, IL6, and IL1 [6–12]. A pleiotropic function for MK2 is revealed through both MK2−/− cells which show defects in migration [11,12] and MK2–/– mice which show neuroprotection against ischemic injury [8] as well as increased susceptibility to infection by *Listeria monocytogenes* [9] and to inflammatory bowel disease [10]. These phenotypes are likely to result from the misregulation of distinct pathways otherwise requiring appropriate regulation by a number of MK2 binding proteins and substrates.

Several approaches have identified potential physiological substrates and binding proteins for MK2 including adapter and cytoskeletal associated proteins [13,14], transcriptional and translation regulators [15–18], regulatory enzymes [19–21], and RNA binding proteins [22,23]. We chose a two-hybrid approach toward identifying MK2 binding proteins and potential substrates to better understand the pleiotropic function of MK2. We identified: ShcA, a signaling phospho-protein [24], human polyhomeotic 2 (HPH2), a transcriptional regulator [25], and highly similar to smoothelin (HSTS) which is related to the cytoskeletal associated protein smoothelin [26,27], as MK2 binding proteins. Both HPH2 and the long isoform of ShcA, p66^{ShcA}, interact with MK2 in mammalian cells and p66^{ShcA} is a substrate for MK2 in vitro suggesting a biological role for these proteins in MK2 regulated pathways.

2. Materials and methods

2.1. Plasmid construction

MK2, MK2K93R, MK2ΔN (amino acids 41–400), MK2ΔC (amino acids 1–370), MK2Cat (catalytic domain amino acids 48–338), and COT/TPL2 were amplified with N: ggatccccgaattccat and C: ctcgagcggccgc terminal linkers and cloned into pGBKT7 (Clontech) *Nde*I-*Xho*I. HPH2 (amino acids 656–858) was cloned using *Sfi*I and *Xho*I, into pCMV-HA (Clontech). V5-p66^{ShcA} in pCMV-PSORT was purchased from Invitrogen.

2.2. Yeast two-hybrid system

The yeast two-hybrid screen was executed using a human bone marrow MATCHMAKER cDNA library cloned into pACT2 (Clontech) and pre-transformed into yeast strain Y187. PGBKT7-MK2K93R, transformed into yeast strain AH109, was used in a mating strategy according to the MATCHMAKER Library user manual (Clontech). MK2 interacting proteins were tested against pGBKT7, pGBKT7-53, pGBKT7-Lamin (Clontech), and pGBKT7-COT/TPL2 by negative selection. Positive colonies were selected by growth and color on SD -Ade/-His/-Leu/-Trp and SD -Leu/-Trp/X-α-Gal respectively. One sequence each of ShcA, HPH2, and HSTS was identified

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Abbreviations: MK2 and MAPKAP kinase 2, mitogen activated protein kinase-activated protein kinase 2; MAPK, mitogen activated protein kinase; COT/TPL2, tumor progression locus; Hsp27, heat shock protein 27; TNF-α, tumor necrosis factor-α; Shc, src homology and collagen; HPH2, human polyhomeotic 2; HSTS, highly similar to smoothelin; ROS, reactive oxygen species

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as selectively interacting with MK2K93R. MK2 interaction domains were mapped using pGBKT7-MK2, pGBKT7-MK2ΔN, pGBKT7-MK2ΔC, and pGBKT7-MK2Cat.

2.3. Cell culture, antibodies and transfection

The human embryonic kidney HEK-293T and HeLa (ATCC) cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS) at 37°C in humidified air with 5% CO_2. RAW 264.7 cells (ATCC) were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10%. Tagged proteins were detected using anti-HA polyclonal (1:200), anti-Myc monoclonal (1:500) (Clontech), anti-Myc polyclonal (1:200) (Santa Cruz Biotechnology), and anti-V5 monoclonal (1: 5000) (Invitrogen) antibodies. Cellular proteins were detected with anti-actin monoclonal (1:5000) (United States Biological), and anti-phospho-Hsp27 S86 (1:1000) (Cell Signaling Technologies) antibodies.

HEK-293T cells were transfected using a calcium phosphate transfection system (Gibco BRL Life Technologies). HeLa and RAW 264.7 cells were transfected using Lipofectamine-2000 (Invitrogen). HeLa cells were starved in 0.5% (v/v) FCS overnight prior to stimulation with anisomycin (Sigma Aldrich) 2 μg/ml, for 30 min. RAW 264.7 cells were stimulated with LPS (*Salmonella typhosa*, Sigma Aldrich) 50 ng/ml, for 6 h.

2.4. Immunoprecipitation and immunoblots

Cells were lysed in Triton lysis buffer (TLB): 20 mM Tris pH 7.5, 125 mM NaCl, 1% (v/v) Triton X-100, 100 mM NaF, 25 mM sodium β-glycerophosphate, 10 mM sodium vanadate with Complete Protease inhibitor cocktail (Roche Molecular Biochemicals) and after centrifugation, lysate protein concentration was measured (DC Protein Assay, Bio-Rad). Following addition of NuPage LDS sample buffer (Invitrogen) supplemented with 20 mM dithiothreitol (DTT), samples were boiled, centrifuged, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 5–12% acrylamide), and transferred to nitrocellulose (0.45 μm) membranes (Invitrogen). Membranes were processed as in [11]. Incubations with primary phospho-specific antibodies were overnight at 4°C in 5% bovine serum albumin (Sigma-Aldrich) in phosphate buffered saline–0.1% Tween 20. Bound proteins were detected with an ECL detection kit (Amersham Biosciences) and quantitated using the Molecular Imager FX densitometer (Bio-Rad) with Quant One software. For immunoprecipitations, lysates were pre-cleared with protein G Sepharose (Amersham Biosciences) and incubated for 2–6 h with primary antibodies in TLB prior to addition of protein G Sepharose for an additional 2 h at 4°C. After centrifugation the Sepharose matrix was washed twice in TLB and diluted in NuPage LDS sample buffer supplemented with 20 mM DTT.

2.5. Kinase assays

Immunoprecipitates from V5-$p66^{ShcA}$ transfected HEK-293T cells were washed in TLB, RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS), and in kinase buffer (0.5 M Tris pH 7.5, 0.2 M $MgCl_2$). 5 μl of the Sepharose matrix was used as substrate for kinase reactions with recombinant MK2 (50 nM), 1 μM ATP, 0.5 μCi [γ-^{33}P]ATP, 3 mM DTT in 1×kinase buffer for 30 min at room temperature. Reactions were terminated by addition of NuPage LDS sample buffer supplemented with 20 mM DTT and resolved by SDS–PAGE. Phosphorylated substrate was detected using the Molecular Imager FX (Bio-Rad).

2.6. Analysis for secreted TNF-α

Medium from transfected RAW 264.7 cells was assayed for secreted TNF-α using enzyme linked immunosorbent assays (ELISA) (Quantikine M mouse TNF-α immunoassay kit, R&D Systems).

3. Results

3.1. MK2 interacting proteins identified using two-hybrid

Full length catalytically inactive MK2 (MK2K93R) [11] was used in executing the two-hybrid screen because inactive kinases have been reported to bind interacting proteins more stably thereby allowing for stronger transcriptional activation of two-hybrid reporter genes [28]. We identified ShcA, an adapter signaling phospho-protein, HPH2, a transcriptional regulator, and HSTS, which is highly related to the cytoskeletal associated protein smoothelin, as binding proteins for MK2 (Fig. 1).

We tested ShcA, HPH2, and HSTS for their interaction with empty vector expressing the GAL4 activation domain alone and three unrelated proteins: p53, lamin, and COT/TPL2. Since there was no detectable interaction between these proteins their interaction with MK2 is specific. Additionally,

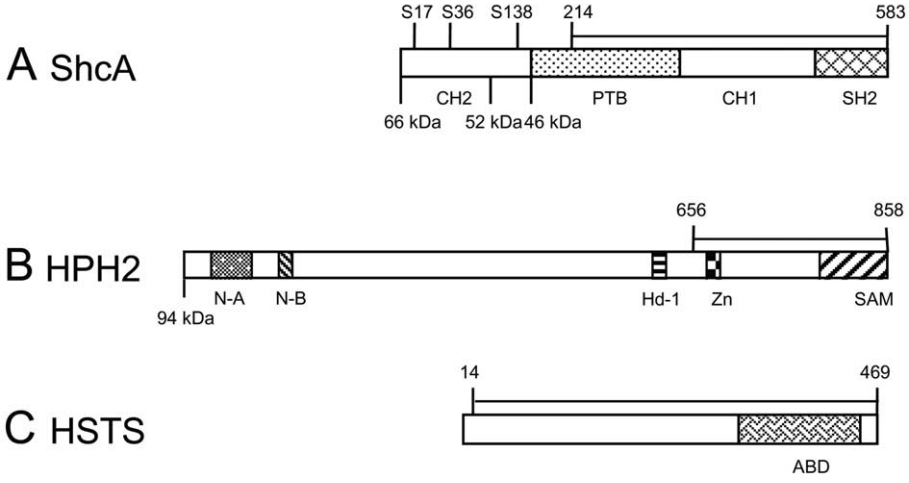

Fig. 1. MK2 interacting proteins identified using two-hybrid. A–D: ShcA, HPH2, and HSTS interact with MK2 in yeast cells. Horizontal lines indicate MK2 interaction domains identified from the screen. A: Amino acid designations in ShcA correspond to the longest isoform: $p66^{ShcA}$, 66 kDa, 52 kDa, and 46 kDa. Domain delineations are (A) CH1 and 2: collagen homology domains 1 and 2, PTB: phospho-tyrosine binding domain, and SH2: Src homology domain; (B) N-A and N-B: conserved N-terminal domains, Hd-1: homology domain I, Zn: zinc coordination domain, SAM: sterile α motif; (C) ABD: actinin type actin binding domain. A: Serine phosphorylation sites in ShcA: S36 and S138, predicted MK2 and CaM-KII consensus site: S17. D: Comparison of HSTS, unnamed protein product *Mus musculus* (Mus), and smoothelinLc2 (smoLc2) using ClustalW. Black and gray shading indicate identical and similar amino acids respectively. Sequences were identified with BLAST database searches and downloaded from GenBank (accession numbers are gi21751336 for HSTS, gi12844213 for unnamed protein *Mus musculus*, and gi8119288 for smoLc2). SmoSc2 start codon at aa 457 (solid arrow), C-terminal homology with HSTS begins at aa 545 (dashed arrow), actinin type actin binding domain (dotted line).

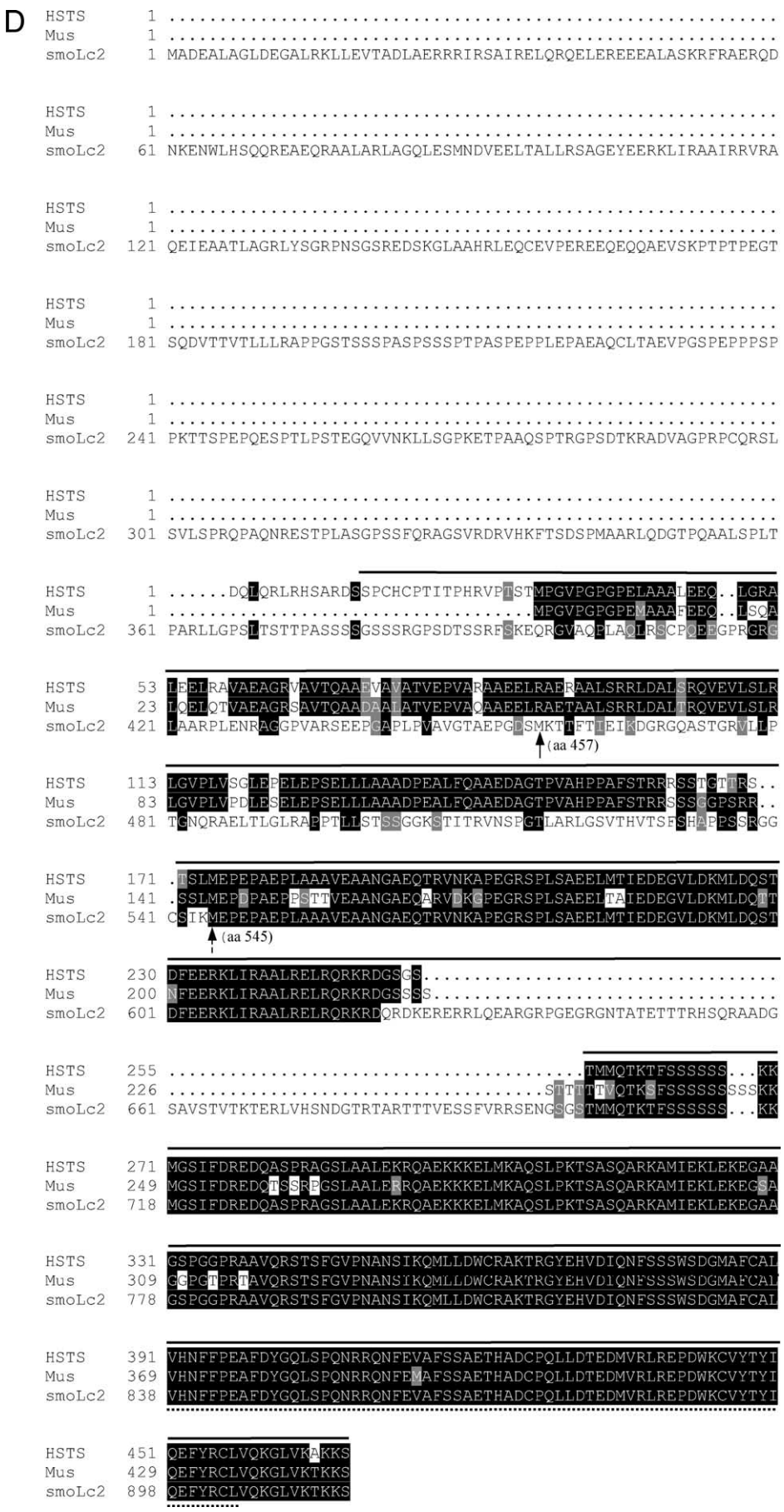

Fig. 1 (*Continued*).

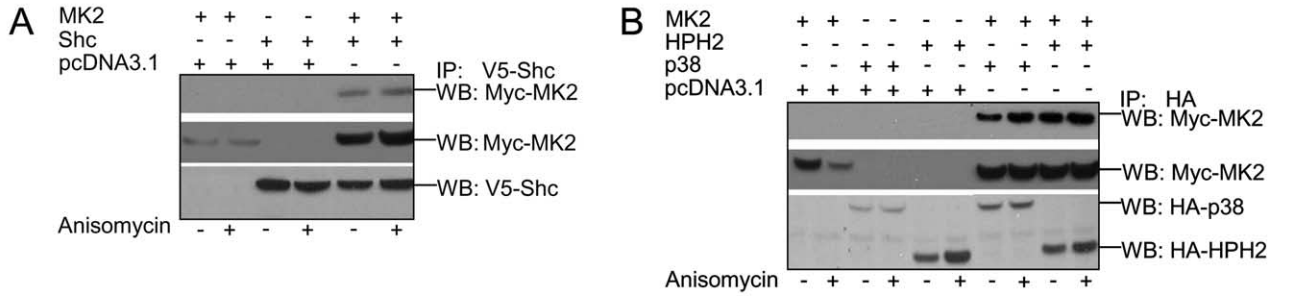

Fig. 2. MK2 co-immunoprecipitates with p66^{ShcA}, HPH2, and p38 expressed in 293T cells. A,B: V5-p66^{ShcA}, HA-HPH2, HA-p38, and Myc-MK2 were expressed in 293T cells. Cell lysate protein levels were quantitated and equal amounts of protein were analyzed. Co-immunoprecipitation using anti-V5 or anti-HA antibodies followed by immunoblotting with an anti-Myc antibody shows that MK2 co-immunoprecipitates (A) with p66^{ShcA} and (B) with HPH2, and p38. Immunoprecipitation (IP), Western blot (WB).

all three MK2 binding proteins bound MK2 and MK2K93R equally, therefore their binding is not an artifact of the kinase inactive mutant MK2K93R. Using several MK2 deletion mutants, we found that the catalytic domain of MK2 (aa 48–338) is sufficient to confer the interaction with all three proteins (data not shown).

3.2. *MK2 co-immunoprecipitates with p66^{ShcA} and HPH2 in mammalian cells*

The interaction of ShcA and HPH2 with MK2 in mammalian cells was confirmed using co-immunoprecipitation. We used a construct encoding the longer ShcA isoform, p66^{ShcA}, in this and in subsequent experiments to analyze the interaction between MK2 and ShcA since p66^{ShcA} is regulated by serine phosphorylation and like the p38 pathway, mediates cellular responses to stress [24,29]. Myc-tagged MK2 (Myc-MK2) was co-expressed with either V5-tagged p66^{ShcA} (V5-p66^{ShcA}), HA-tagged HPH2 (HA-HPH2), or HA-tagged p38 (HA-p38) as a positive control in 293T cells. Immunoprecipitation of cell lysates with anti-V5 or anti-HA antibodies and subsequent immunoblotting using an anti-Myc antibody to detect MK2 showed that all three proteins co-immunoprecipitate with MK2 (Fig. 2A,B). Quantitative immunoblot analyses show increased MK2 levels with p66^{ShcA}, HPH2, or p38 co-expression indicating that MK2 stability increases with binding. P38 levels increase with MK2 co-expression suggesting that p38 stability also increases when co-expressed with MK2. The interaction of MK2 with p66^{ShcA}, HPH2, and p38 appears to be constitutive since activation of MK2 with anisomycin ([18,30]; unpublished results), does not affect the formation of these complexes as assayed by co-immunoprecipitation (Fig. 2).

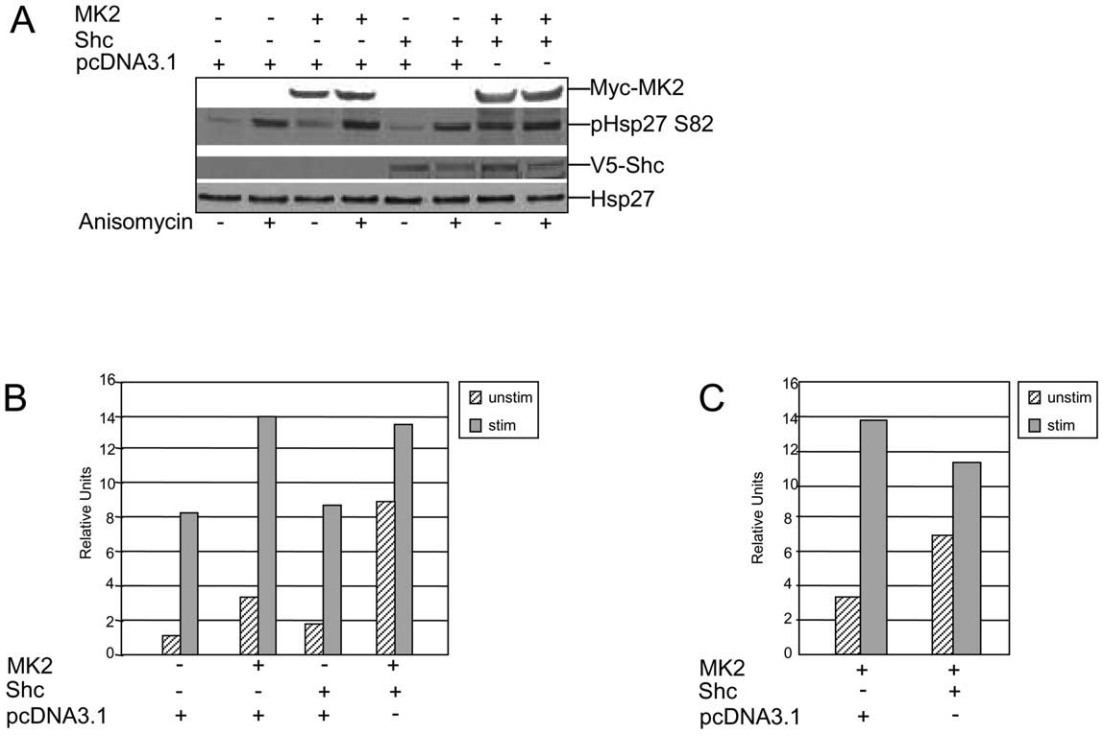

Fig. 3. MK2 is activated with p66^{ShcA} co-expression in HeLa cells. A: V5-p66^{ShcA} and Myc-MK2 were expressed in HeLa cells. Cell lysate protein levels were quantitated and equal amounts of protein were analyzed using immunoblotting. Phospho-Hsp27 increases with anisomycin treatment and with MK2 expression in unstimulated cells. A further increase in phospho-Hsp27 is observed with co-expression of MK2 and p66^{ShcA}. B,C: Phospho-Hsp27 normalized to basal phospho-Hsp27 levels in unstimulated vector control transfected cells (B) and normalized with Myc-MK2 expression levels (C).

3.3. MK2 is activated when co-expressed with p66^{ShcA} in HeLa cells

Phosphorylation of endogenous Hsp27, a physiological substrate for MK2 [6,31], is responsive to MK2 activity in HeLa cells (unpublished results) and therefore was used to assay for MK2 activity when co-expressed with p66^{ShcA}. HeLa cells were transfected with vector alone, V5-p66^{ShcA}, or Myc-MK2 as controls or were co-transfected with both V5-p66^{ShcA} and Myc-MK2. Subsequent immunoblotting for phosphorylated Hsp27 (phospho-Hsp27) shows an increase in phospho-Hsp27 with anisomycin, a stimulus which is known to activate the p38/MK2 pathway in this cell type ([17]; unpublished results). In unstimulated cells an increase in basal phospho-Hsp27 levels is apparent with expression of exogenous MK2, which is not observed in p66^{ShcA} or vector control expressing cells (Fig. 3A). Densitometry shows the increase in basal phospho-Hsp27 seen with MK2 expression to be two-fold over empty vector or p66^{ShcA} expressing cells. Basal phospho-Hsp27 levels are further increased when MK2 is co-expressed with p66^{ShcA}. Densitometry shows this increase to be three-fold over levels detected when MK2 is expressed alone (Fig. 3B). As shown in 293T cells, MK2 levels increase with co-expression of p66^{ShcA} further demonstrating their interaction. The observed increase in basal phospho-Hsp27 is therefore likely to reflect increased MK2 activity as well as increased levels of MK2 protein. The basal phospho-Hsp27 level in cells co-expressing MK2 and p66^{ShcA} is 2.3-fold over the phospho-Hsp27 level in control transfected cells when normalized with MK2 protein expression (Fig. 3C) and is likely the result of increased MK2 activation with p66^{ShcA} co-expression.

3.4. MK2 is activated when co-expressed with p66^{ShcA} in RAW 264.7 cells

To further confirm that MK2 is activated with p66^{ShcA} binding, we assayed for secreted TNF-α from RAW 264.7 cells co-expressing MK2 and p66^{ShcA}. RAW 264.7 cells are of macrophage origin and express the cell surface receptor CD14 thereby conferring their responsiveness to LPS measured here by TNF-α release. TNF-α regulation through MK2 is known to require MK2 enzymatic activity [11], therefore increased TNF-α levels with MK2 and p66^{ShcA} co-expression should reflect MK2 activation. RAW 264.7 cells were transfected with vector alone, V5-p66^{ShcA}, or Myc-MK2 alone as controls or were co-transfected with both V5-p66^{ShcA} and Myc-MK2. TNF-α ELISAs showed that TNF-α secretion increases eight-fold with LPS stimulation and that expression of MK2 or p66^{ShcA} alone does not potentiate TNF-α biosynthesis. In contrast, when MK2 and p66^{ShcA} are co-expressed, TNF-α biosynthesis increases 1.5-fold after stimulation (Fig. 4A).

Quantitative Western blot analysis shows that both Myc-MK2 and V5-p66^{ShcA} are expressed in RAW 264.7 cells although in contrast to in 293T and in HeLa cells, both Myc-MK2 and V5-p66^{ShcA} levels are lower in co-transfected versus control transfected cells. This does not reflect an overall decrease in protein levels because equal amounts of protein were loaded in each lane as shown using an actin specific antibody (Fig. 4B). The increase in TNF-α biosynthesis therefore should reflect an increase in MK2 activity and not in MK2 expression since MK2 protein levels when co-expressed with p66^{ShcA} are below that observed in cells expressing MK2 alone.

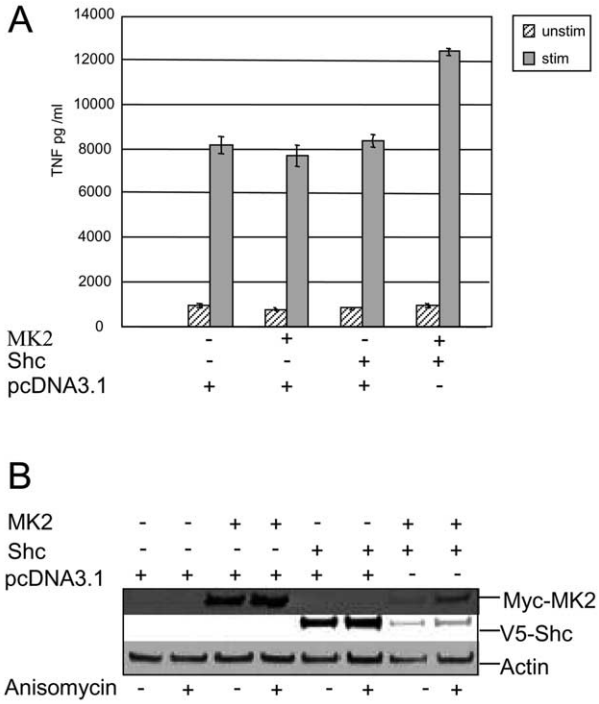

Fig. 4. MK2 is activated with p66^{ShcA} co-expression in RAW 264.7 cells. A: ELISA analysis shows an increase in TNF-α secretion when MK2 and p66^{ShcA} are co-expressed. B: V5-p66^{ShcA} and Myc-MK2 were expressed in RAW 264.7 cells. Cell lysate protein levels were quantitated and equal amounts of protein were analyzed as shown with an anti-actin specific antibody.

3.5. MK2 phosphorylates p66^{ShcA} in vitro

Serine 36 phosphorylation is required for p66^{ShcA} function in mediating cellular responses to oxidative stress [24]. A predicted recognition sequence for both MK2 and calcium/calmodulin dependent protein kinase-II (CaM-KII) is located at serine 17: RXXS, although the preferred recognition site for MK2 includes a hydrophobic amino acid at N-5: hydrophobic aa-Xaa-Arg-Xaa-Xaa-Ser [32]. To determine if p66^{ShcA} is a substrate for MK2, V5-p66^{ShcA} was expressed in 293T cells. After expression, p66^{ShcA} was immunoprecipitated using an anti-V5 antibody and used as substrate with activated recombinant MK2 in an in vitro kinase assay. When compared with vector control transfected cells, immunoprecipitates from p66^{ShcA} transfected cells show robust phosphorylation at 66 kDa demonstrating that p66^{ShcA} is a substrate for MK2 in vitro (Fig. 5).

4. Discussion

A two-hybrid approach using an oligo-dT primed library generated from human bone marrow identified ShcA, HPH2, and HSTS as MK2 specific interacting proteins (Fig. 1). The interaction of MK2 with the longest isoform of ShcA, p66^{ShcA}, and HPH2 amino acids 656–858 was confirmed using co-immunoprecipitation. MK2 protein levels increase with co-expression of p66^{ShcA}, HPH2, or p38 in 293T or in HeLa cells, most likely due to increased MK2 stability with binding (Figs. 2 and 3), further demonstrating their interaction. P38 levels also increase with co-expression of MK2 (Fig. 2B) supporting a previously reported role for MK2 in stabilizing p38 levels through binding [11].

HPH2 belongs to the polycomb group of proteins, which maintain appropriate regulation of the homeotic loci through the formation of chromatin associated complexes. Mutations at these loci result in anterior–posterior transformations [33], defects in neural crest and heart development, and severe hypoplasia in the thymus and spleen suggesting that these proteins regulate hematopoiesis [34,35]. The hematopoietic cell type specific defect in mice deleted for MK2 is illustrated by a dramatic decrease in the production of a number of pro-inflammatory cytokines, largely due to a misregulation in processing or translation of their mRNAs [6,7]. As yet, a defect in hematopoietic cell development has not been revealed in mice deleted for MK2, therefore a biological role for MK2 in binding HPH2 has yet to be elucidated and may also include a role in modulating HPH2's regulation of the homeotic loci.

The HSTS gene predicted amino acid sequence shows highest identity, 82%, with unnamed protein product *Mus musculus* but extends 31 amino acids N-terminal to the murine protein's start codon, indicating that this murine sequence is incomplete or is a truncated isoform of HSTS (Fig. 1D). The amino acid sequence of HSTS reported in GenBank is also likely incomplete since it does not begin with a methionine start codon (Fig. 1D). HSTS and unnamed protein product *Mus musculus* are related to the smoothelin family of cytoskeletal associated proteins. Small and large (L) smoothelin isoforms (Fig. 1D) are generated through distinct transcription initiation sites with additional isoforms, c1–c3, generated through C-terminal alternative splicing [26,27]. HSTS is most similar to smoothelinLc2 sharing highest identity, 97%, from aa 545 through the conserved actinin type actin binding domain with the exception of a 76 aa deletion in this region (Fig. 1D). N-terminal to aa 545 the homology between HSTS and smoothelinLc2 lessens significantly representing previously unreported divergence within the smoothelinL family. Of note, smoothelinLc2 is specifically expressed in vascularized tissues such as smooth muscle and heart where MK2 is also highly expressed [36]. The function of the smoothelin proteins is unknown, however, their association with actin suggests that HSTS may localize MK2 to the cytoskeleton to regulate microfilament dynamics possibly by phosphorylating its substrate Hsp27. This possibility is further supported by the cellular migration defect shown by cells deleted for MK2, a phenotype attributed to reduced phosphorylation of an F-actin cross-linking protein [12] or misregulation of actin polymerization [11] in the absence of MK2.

The ShcA locus encodes three Shc isoforms p46^{ShcA}, p52^{ShcA}, and p66^{ShcA}. P46^{ShcA} and p52^{ShcA} activate the Ras MAPK pathway through cell surface receptors while p66^{ShcA} is proposed to down-regulate signaling through its competitive binding with p46^{ShcA} and p52^{ShcA} [37,38]. Unlike the smaller isoforms of ShcA, p66^{ShcA} also regulates oxidative stress responses and is regulated by serine phosphorylation [24,29]. MK2 interacts with the ShcA C-terminus shared by all three ShcA isoforms, therefore MK2 should interact with both p46^{ShcA} and p52^{ShcA} in mammalian cell based assays. Although MK2 has not been associated with signaling directly through cell surface receptors, binding or phosphorylation by MK2 could regulate p46^{ShcA} and p52^{ShcA} through a yet to be described mechanism.

The HeLa and RAW 264.7 cell assays provide a biological context in which to evaluate MK2 activity through two MK2 regulated responses, Hsp27 phosphorylation and TNF-α release [6]. Co-expression with p66^{ShcA} activates MK2 as shown by increased HeLa cell phospho-Hsp27, which is apparent even when normalized with MK2 levels (Fig. 3). Since MK2 levels increase with p66^{ShcA} co-expression, the observed increase in MK2 activity is likely to result from both increased protein stability and kinase activity. P66^{ShcA} over-expression itself may also indirectly activate MK2 by increasing basal levels of intracellular ROS [29,39] which then stimulate the p38 stress activated protein kinase cascade [4].

MK2 activation with p66^{ShcA} co-expression, as assayed by increased TNF-α secretion from RAW 264.7 cells, further illustrates the interaction between these proteins, and is revealed only after stimulation. MK2 localization studies have shown that when activated, MK2 translocates from the nucleus to the cytoplasm [30,40,41]. Therefore, in RAW 264.7 cells, activated MK2 may become tethered in the cytoplasm through its interaction with cytosolic p66^{ShcA} where it can better access cytoplasmic substrates which regulate the biosynthesis of TNF-α mRNAs [23]. The observed increase in TNF-α secretion may also result directly from increased MK2 activation with p66^{ShcA} binding as observed in HeLa cells. The reduced expression of both MK2 and p66ShcA in RAW 264.7 cells when co-transfected may result from lowered transfection efficiency or degradation upon co-expression in this cell type.

Our finding that p66^{ShcA} is a direct in vitro substrate for MK2 suggests that MK2 regulates p66^{ShcA} activity (Fig. 5). Studies including site directed mutagenesis are under way to identify which p66^{ShcA} amino acid is phosphorylated by MK2. Resistance to stress induced cellular apoptosis and an extended lifespan, both attributed to lowered levels of intracellular ROS, characterize animals deleted for p66^{ShcA} [24]. To date, an extended lifespan has not been reported for animals deleted for MK2. However, MK2−/− mice do have enlarged germinal centers which are attributed to decreased apoptosis in the cells that comprise these tissues [6]. This phenotype may be due to misregulation of p66^{ShcA} in the absence of MK2. Reduced phospho-Akt levels, also resulting from lowered levels of intracellular ROS, have been reported for p66$^{ShcA-/-}$ Mefs [38]. Although phosphorylation of Akt by MK2 has been reported in vitro [21], MK2 regulation of Akt in cells, has yet to be determined.

In summary, using yeast two-hybrid and subsequent co-immunoprecipitation we have identified three MK2 interacting proteins: ShcA, HPH2 and HSTS. The interaction of MK2 with p66^{ShcA} activates MK2 as shown by the increased phosphorylation of its physiological substrate Hsp27, and an in-

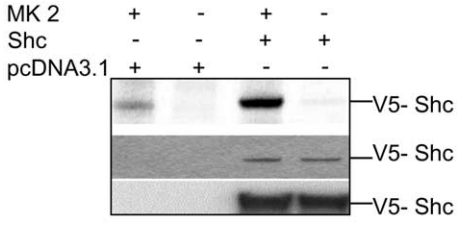

Fig. 5. MK2 phosphorylates p66^{ShcA} in vitro. 293T cells were transfected with V5-p66^{ShcA} to generate substrate or with empty vector as a control. Coomassie staining (middle panel) and immunoblotting using an anti-V5 antibody (lower panel) show that p66^{ShcA} is expressed. The anti-V5 antibody immunoprecipitates an endogenous protein from 293T cells that is a weak substrate for MK2. Robust phosphorylation at 66 kDa is only observed with immunoprecipitates from cells expressing V5-p66^{ShcA}.

creased level of LPS induced TNF-α production, an MK2 mediated response [6]. The phosphorylation of $p66^{ShcA}$ by MK2 further supports a biological role for the interaction of MK2 with $p66^{ShcA}$ in cells. Future experiments are needed to further understand the precise mechanism underlying these interactions and to determine a physiological role for MK2 interacting proteins in MK2 regulated pathways.

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