Cooperation of ERK and SCF\textsuperscript{Skp2} for MKP-1 Destruction Provides a Positive Feedback Regulation of Proliferating Signaling\textsuperscript{*}

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The dual-specificity MAPK phosphatase MKP-1/CL100/DUSP1 is an inducible nuclear protein controlled by p44/42 MAPK (ERK1/2) in a negative feedback mechanism to inhibit kinase activity. Here, we report on the molecular basis for a novel positive feedback mechanism to sustain ERK activation by triggering MKP-1 proteolysis. Active ERK2 docking to the DEF motif (FXFP, residues 339–342) of N-terminally truncated MKP-1 in vitro initiated phosphorylation at the Ser\textsuperscript{296}/Ser\textsuperscript{323} domain, which was not affected by substituting Ala for Ser at Ser\textsuperscript{359}/Ser\textsuperscript{364}. The DEF and Ser\textsuperscript{296}/Ser\textsuperscript{323} sites were essential for ubiquitin-mediated MKP-1 proteolysis stimulated by MKK1-ERK signaling in H293 cells, whereas the N-terminal domain and Ser\textsuperscript{359}/Ser\textsuperscript{364} sites were dispensable. ERK activation by serum increased the endogenous level of ubiquitinated phospho-Ser\textsuperscript{296} MKP-1 and the degradation of MKP-1. Intriguingly, active ERK-promoted phospho-Ser\textsuperscript{296} MKP-1 bound to SCF\textsuperscript{Skp2} ubiquitin ligase in vivo and in vitro. Forced expression of Skp2 enhanced MKP-1 polyubiquitination and proteolysis upon ERK activation, whereas depletion of endogenous Skp2 suppressed such events. The kinetics of ERK signaling stimulated by serum correlated with the endogenous MKP-1 degradation rate in a Skp2-dependent manner. Thus, MKP-1 proteolysis can be achieved via ERK and SCF\textsuperscript{Skp2} cooperation, thereby sustaining ERK activation.

Members of the family of mitogen-activated protein kinases (MAPKs),\textsuperscript{2} including extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38 kinases, are important intracellular signaling molecules regulated by phosphorylation in response to a wide variety of extracellular stimuli such as growth factors and environmental stresses (1–4). Activation of MAPKs requires dual phosphorylation by specific MAPK kinases (MKKs) at the Thr and Tyr residues of the TXY sites in the activation loop, and dephosphorylation of these residues by protein phosphatases, including the dual-specificity MAPK phosphatases (MKPs/DUSPs), terminates such activation (5, 6). Each activated MAPK specifically targets a variety of proteins such as downstream kinases and transcription factors that regulate expression of particular genes and their activators/modulators and phosphatases whose feedback turns off the signaling. The strength and duration of MAPK activation, as well as the stimulus and cell type, distinctly affect cellular outcomes such as cell cycle progression and cell proliferation, differentiation, survival, and apoptosis (7, 8). Activation and inactivation of MAPKs must therefore be tightly controlled with high specificity and efficiency to achieve appropriate cellular responses.

The identification of specific docking domains in non-catalytic regions of MAPKs and their interacting proteins provides a principal mechanism for controlling signaling specificity and efficiency (9, 10). Most MAPK-interacting proteins have a conserved domain called the kinase interaction motif (KIM) or D/\delta kinase-docking domain containing a cluster of basic residues followed by an LXX site or surrounded by hydrophobic amino acids (9, 10). The KIM domain exhibits high affinity interactions with an acid-rich region termed the common docking domain in the C termini of all three MAPKs (11). Differences in the amino acid sequences of these common docking and KIM domains in the MAPKs and their substrates further fine-tune docking specificities (9, 10). A second MAPK docking site called the docking site for ERK, FXFP (DEF) motif was originally found in the LIN-1/AOP/Elik-1/SAP-1 subfamily of ETS transcription activators (12, 13). Upon activation, phospho-ERK2 exposes a hydrophobic pocket for DEF motif interactions (14). The DEF motifs and KIM domains can function independently or additively in directing distinct MAPKs to phosphorylate transcription factors at a specific Ser or Thr residue followed by Pro ((S/T)P) (15–19).

All mammalian MKPs identified thus far contain an N-terminal KIM domain and a phosphatase active-site HC\textsubscript{X}C\textsubscript{X}C\textsubscript{R} motif (5, 6, 10). Among them, MKP-1/CL100/DUSP1 and MKP-3/Pyst1/DUSP6 are prototypes whose functional associations with MAPK signals have been well characterized. MKP-1 is a nuclear enzyme encoded by an intermediate-early gene (IEG), and its transcription is rapidly induced in response to many of the stimuli that activate MAPKs (20, 21). By contrast, MKP-3 is localized mainly in the cytosol, and its expression is not inducible by either mitogens or cellular stresses (22, 23), but can be stimulated by agents promoting neuronal differentiation (23). MKP-3 phosphatase activity is allosterically elevated upon selective binding to ERK2 in vitro (24), which may explain the highly specific inactivation of ERK by MKP-3 in vivo (22). Likewise, catalytic activation of MKP-1 is mediated via physical interactions with ERK2, JNK1, and p38α in vitro (25); this also correlates with the substrate specificity of MKP-1 in vivo (21, 25, 26). Thus, distinct MAPKs can elicit a negative feedback loop to control signaling strength and duration.

MKP-1 is rapidly degraded soon after its induction by mitogens in rodent fibroblasts (27, 28). Similarly, in oocytes, Xenopus CL100 (an MKP-1 homolog) is a labile protein, and sorbitol further enhances its destruction (29). MKP-1 is accumulated upon proteasome inhibition in...
MKP-1 Proteolysis via ERK and SCF<sub>Skp2</sub>

FIGURE 1. The DEF motif and its adjacent amino acids in MKP-1. Human MKP-1 has a putative DEF motif at residues 339–342 with four neighboring SP sites: Ser<sup>340</sup>, Ser<sup>341</sup>, Ser<sup>342</sup>, and Ser<sup>343</sup> (black dots). The KIM domain and the phosphatase catalytic domain (CAD) in MKP-1 are also illustrated.

FIGURE 2. Mutated DEF motif in MKP-1 impairs active ERK2 docking and phosphorylation in vitro. A, glutathione-Sepharose bead-attached GST-ERK2 unactive or active) was mixed with ΔN-MKP-1 (+) or ΔN-MKP-1(ANAP) (upper panels). Alternatively, bead-attached GST-ΔN-MKP-1 or GST-ΔN-MKP-1(ANAP) was mixed with unactive or active ERK2 (lower panels). The mixtures were kept at 4°C for 3 h, and protein binding was resolved by GST pull-down and subsequent Western blotting using the indicated antibodies. Equal amounts of proteins used for the pull-down assay were also analyzed by Western blotting (indicated as loading). B, bead-attached GST-tagged active ERK2 was reacted with ΔN-MKP-1 (+) or ΔN-MKP-1(ANAP) in the presence of 20 μM ATP and 1 μCi of [γ-<sup>32</sup>P]ATP for 30 min at 30°C. The samples were then analyzed by electrophoresis, followed by autoradiography (indicated as Kinase assay). A portion of the bead-attached GST-tagged active ERK2 was analyzed by Western blotting to determine equal input. Kinase assay results were calculated by averaging four independent experiments. The Western blots shown are representative of four experiments.

rodent fibroblasts (28). Following removal of the proteasome inhibitory stress, MKP-1 is rapidly degraded, and forced expression of the ERK signal leads to MKP-1 phosphorylation and a decrease in degradation (28). In sorbitol-treated oocytes, forced activation of the ERK2 signal leads to MKP-1 phosphorylation and a decrease in degradation (29). On the other hand, upon Pb(II) exposure or overexpression of constitutively active MKK1/2 in several mammalian cell lines, ERK activation triggers MKP-1 degradation via the ubiquitin-proteasome pathway, indicating a positive feedback control (30). The concluding suggests that ERK may exhibit dual roles in controlling MKP-1 stability in distinct cellular environments. This has driven us to explore the molecular basis for ERK-directed MKP-1 ubiquitination and to establish whether such an event also occurs during serum stimulation of quiescent cells. Here, we show that the MKP-1 DEF motif is necessary for active ERK2 binding to initiate site-specific phosphorylation, serving as an essential recognition domain for the Skp1/Cul1/F-box protein Skp2 (SCF<sub>Skp2</sub>) ubiquitin-protein isopeptide ligase (E3), a vital E3 enzyme for S phase entry and progression (31, 32), leading to MKP-1 polyubiquitination and subsequent destruction via the 26 S proteasome. These results suggest that active ERK docking to the DEF motif and SCSk<sub>2</sub> association are rate-limiting for the ubiquitin-mediated MKP-1 proteolysis that would sustain ERK signaling to facilitate G<sub>1</sub> cells entering the cell cycle.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The plasmid pcSG5-MKP-1-Myc was kindly provided by Dr. N. K. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Forward and reverse primers containing EcoRV and HindIII restriction sites were used to amplify MKP-1 cDNA from pcSG5-MKP-1-Myc by PCR. This fragment was subcloned in pCMV-Tag2B (Stratagene, La Jolla, CA) to generate a FLAG-tagged wild-type (WT) MKP-1 expression vector. Similarly, the...
plasmids expressing glutathione S-transferase (GST)-tagged WT (GST-MKP-1) and N-terminally truncated (amino acids 1–59; GST-ΔN-MKP-1) MKP-1 cDNAs were constructed by subcloning a PCR-amplified BamHI-EcoRI fragment from pSG5-MKP-1-Myc in pGEX-4T-1 (Amersham Biosciences). Various MKP-1 and ΔN-MKP-1 substitution mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). All PCR-amplified fragments or mutagenized constructs were verified by DNA sequencing using the BigDye terminator cycle reaction kit and an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

**Production of Recombinant Proteins**—The vectors encoding GST-tagged WT MKP-1 and mutants were expressed in *Escherichia coli* BL21. The GST fusion proteins were purified via binding to glutathione-Sepharose 4B (Amersham Biosciences), followed by elution in buffer containing 10 mM HEPES (pH 7.5), 15 mM glutathione, 150 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin.

**In Vitro Binding and Phosphorylation**—Equal amounts (0.2 μg) of inactive and active forms of GST-ERK2 fusion proteins (Upstate Biotechnology, Inc., Lake Placid, NY) were reacted overnight with glutathione-Sepharose beads at 4 °C. Various GST-ΔN-MKP-1 fusion proteins (2 μg) were treated with thrombin (0.04 units) to release GST fragments and allowed to interact with bead-attached GST-ERK2 fusion proteins at 4 °C for 3 h in binding buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride). Similarly, inactive and active forms of ERK2 were reacted with bead-attached GST-ΔN-MKP-1 mutants. Following three washes with 50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol, the associated proteins were released by boiling the beads for 5 min and resolved by SDS-PAGE, followed by Western blotting.

Bead-attached GST-tagged active ERK2 was also reacted with various ΔN-MKP-1 mutants and 20 mM ATP in the presence or absence of 1 μCi of [γ-32P]ATP. The phosphorylation reaction was performed at 30 °C for 30 min in a total volume of 30 μl of kinase reaction buffer (20 mM HEPES (pH 7.6), 20 mM MgCl2, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 1 mM NaF). Next, the reaction mixture was boiled, and the phosphorylated protein was resolved by SDS-PAGE, followed by either autoradiography or Western blotting using anti-phospho-Ser296 MKP-1 antibody. The amount of GST-tagged active ERK2 in the reaction mixture was determined by Western blotting.

**Cells, Vectors, Small Interfering RNA (siRNA), and Transfection**—The human embryonic kidney cell line H293 was cultured in Dulbecco’s minimal essential medium containing 10% fetal calf serum as described previously (30). Vectors expressing MKK1-CA (a constitutively active form of MKK1, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) were gifts from Dr. N. G. Ahn (University of Colorado, Boulder, CO). pcDNA-FLAG-Skp2 was kindly provided by Dr. M. Pagano (New York University School of Medicine, New York, NY). The sense strand sequences of siRNA duplexes used for Skp2 and lamin (as a control) were 5′-AAUGC-UAAGCCUGGAAGGCCUCGdTdT-3′ and 5′-CUGGACUUCCAGAGAACAdTdT-3′ (Drharmacon, Inc. Lafayette, CO). The expression vectors were transfected into H293 cells using Lipofectamine (Invitrogen). The siRNA duplexes (200 nM) were transferred into cells immediately before and 24 h after vector transfection using Oligofectamine (Invitrogen). The transfected vectors were allowed 2 days of expression in the presence or absence of siRNAs, and the whole cell extract (WCE) was subjected to immunoprecipitation and/or Western blotting. To reveal ubiquitinated proteins, 10 μM N-acetyl-Leu-Leu-norleucinal (ALLN; Calbiochem-Novabiochem), an inhibitor of the 26S proteasome, was supplied for the final 3 h during transfection.

**Serum Stimulation**—Cells were cultured in medium containing 0.1% serum for 24 or 48 h and then treated with 10–20% serum for 15 min to 3 h. To prevent *de novo* protein synthesis, 10 μg/ml cycloheximide (Sigma) was added to cells during serum stimulation. To reveal phosphorylated and ubiquitinated MKP-1, 10 μM ALLN was supplied for the final 10 min or 1 h during serum stimulation. To determine the effect of ERK activation on MKP-1 degradation, U0126 (Calbiochem-Novabiochem), a specific MMK1/2 inhibitor, was added 1 h before serum stimulation.

**Protein Stability Analysis**—The half-lives of endogenous and exogenous MKP-1 proteins were determined using pulse-chase experiments. Serum-starved cells were cultured in methionine-free medium for 30 min and then pulse-labeled with 5 μCi/ml [35S]methionine for 1 h in the presence or absence of U0126 (5 μM). Immediately after labeling, the cells were washed with a chasing medium containing 30 μg/ml methionine prior to stimulation with 10% serum in the chasing medium for 0–3 h. Cells were then lysed, and MKP-1 proteins in the WCE were subjected to immunoprecipitation and subsequent electrophoresis. Isootope-labeled MKP-1 was visualized by autoradiography.

**Western Blot Analysis**—WCE collection was performed as described previously (33). The BCA protein assay kit (Pierce) was employed to determine protein concentrations using bovine serum albumin as a standard. Equal amounts of proteins in the WCE were fractionated by SDS-PAGE. The protein bands were then transferred electrophoretically to polyvinylidene difluoride membranes and probed with primary antibody, followed by a horseradish peroxidase-conjugated secondary antibody. The anti-phospho-Thr202/Tyr204 ERK1/2 polyclonal antibody was from Cell Signaling Technology, Inc. (catalog no. 9101; Beverly, MA).
MA). The polyclonal antibodies against ERK2 (C-14), MKP-1 (V-15), ubiquitin (P4D1), Skp2 (H-435), hemagglutinin (HA; F-7), Skp1 (H-163), Cul1 (H-213), Cks1 (FL-79), p27 (C-19), and α-tubulin (TU-02) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG monoclonal antibody M2 was from Sigma. The anti-phospho-Ser296 MKP-1 antibody was generated from rabbits injected with phosphopeptide KQRRSIIpSPNFSFMG conjugated to keyhole limpet hemocyanin. Antibodies were stripped from polyvinylidene difluoride membranes using a solution containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 0.7% (w/w) β-mercaptoethanol at 50 °C for 15 min before reprobing with another primary antibody. Relative protein blot intensities were determined using a computing densitometer equipped with the ImageQuant analysis program (Amersham Biosciences).

**Immunoprecipitation**—Cells were washed twice with ice-cold phosphate-buffered saline and harvested at 4°C in immunoprecipitation lysis buffer as described previously (30). Equal amounts of proteins were immunoprecipitated using antibodies against ubiquitin, FLAG, Skp2, ERK2, MKP-1, and phospho-Ser296 MKP-1 and collected with protein G-Sepharose beads at 4°C for 16 h. The immunoprecipitate was then washed three times with cold lysis buffer and subjected to Western blotting.

**In Vitro Ubiquitination**—The SCFSkp2-Cks1 E3 complex was prepared as follows. The FLAG-Skp2 vector was transfected into H293 cells and allowed expression for 2 days, and the WCE was immunoprecipitated using anti-FLAG antibody. The FLAG-Skp2 immunocomplex was then mixed with equal amounts of the Cks1 immunocomplex prepared separately from H293 cells. Following the phosphorylation reaction, WT MKP-1 and mutants (5 μg) were subjected to in vitro ubiquitination assay using ubiquitin-activating enzyme (E1; 0.5 μg; AFFINITI Research Products Ltd., Plymouth Meeting, PA), UbcH3 conjugating enzyme (1 μg; AFFINITI), SCFSkp2-Cks1 immunocomplex, and ubiquitin (50 μg; AFFINITI) in a reaction buffer containing 40 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM dithiothreitol, 10% (v/v) glycerol, 10 mM phosphocreatine, 100 μg/ml creatine kinase, 0.5 mM ATP, 1 mM 4-(2-amino-
MKP-1 Proteolysis via ERK and SCF^{Skp2}

**FIGURE 6.** Serum decreases the half-lives of WT MKP-1 and S359A/S364A in H293 cells at a rate noticeably faster than that occurring in ANAP and S296A/S323A. Following transfection with FLAG-tagged WT MKP-1 or its derivatives ANAP, S296A/S323A, and S359A/S364A, cells were cultured in complete medium for 24 h and starved in 0.1% serum for another 24 h. The cells were then pulse-labeled with [35S]methionine for 1 h prior to stimulation with 10% serum for 0–3 h in a chasing medium. Exogenous MKP-1 proteins were immunoprecipitated using anti-FLAG antibody and then subjected to gel electrophoresis, followed by autoradiography. The chart shows the results from quantitative analyses of the half-lives of FLAG-tagged proteins calculated by averaging three independent experiments.

**RESULTS**

The MKP-1 DEF Motif Is Essential for Active ERK Binding and Phosphorylation, Leading to Ubiquitination and Proteolysis—ERK can induce mpk-1 gene expression at the transcriptional level (34) and enhance phosphatase activity via docking to the N-terminal KIM domain of MKP-1 (25), suggesting a negative feedback loop to inhibit the kinase activity. By contrast, ERK can also trigger MKP-1 proteolysis, reflecting a positive feedback loop to sustain the kinase activity (30).

Given that MKP-1 contains one putative DEF motif (FNFP, residues 339–342) in the C terminus for ERK docking (Fig. 1), we explored whether this motif is essential for ERK-triggered proteolysis. To avoid the possibility of the KIM domain competing with the DEF motif for ERK docking, we constructed the GST-tagged ΔN-MKP-1 mutant, in which the N-terminal 1–59 residues were truncated, and its derivative ΔN-MKP-1(ANAP), in which the two Phe residues in the DEF motif (ENFP) were mutated to Ala. The ΔN-MKP-1 and ΔN-MKP-1(ANAP) fusion proteins were expressed in and purified from bacteria, followed by *in vitro* incubation with either unactive or active ERK2. Fig. 2A shows that ΔN-MKP-1 formed a complex with active ERK2, but ΔN-MKP-1(ANAP) did not; conversely, neither ΔN-MKP-1 nor ΔN-MKP-1(ANAP) associated with inactive ERK2. Furthermore, active ERK2 could phosphorylate ΔN-MKP-1 but not ΔN-MKP-1(ANAP) (Fig. 2B). These *in vitro* results suggest that the MKP-1 DEF motif is essential for active ERK2 docking and phosphorylation.

To explore whether the DEF motif plays a crucial role in MKP-1 ubiquitination stimulated by ERK *in vivo*, we cotransfected FLAG-MKP-1 or FLAG-ΔN-MKP-1 with HA-MKK1-CA into H293 cells, allowed expression for 2 days, and treated one set of cells with an inhibitor of the 26 S proteasome (10 μM ALLN) for the final 3 h to reveal protein ubiquitination. The second set of cells was not treated with ALLN to determine protein degradation. As with FLAG-MKP-1, FLAG-ΔN-MKP-1 was polyubiquitinated and degraded during forced expression of MKK1-CA in H293 cells (Fig. 3, A and B, lanes 7–10), suggesting that the MKP-1 KIM domain is dispensable for ERK-directed ubiquitination *in vivo*. We next constructed a FLAG-tagged full-length MKP-1(ANAP) mutant to evaluate the physiological role of the DEF motif in ERK activation. Forced expression of MKK1-CA in H293 cells caused polyubiquitination in FLAG-MKP-1 but not in the ANAP mutant (Fig. 3A, compare lanes 5 and 6). MKK1-ERK signaling also markedly enhanced a decrease in the FLAG-MKP-1 protein level (Fig. 3B, *first row*, compare lanes 3 and 5), which was notably higher than that in the ANAP mutant level (Fig. 3B, *second row*, compare lanes 4 and 6; and C, *left panel*).

Identification of ERK-directed Phosphoacceptor Sites in MKP-1 for Proteolysis via the Ubiquitin-Proteasome Pathway—Previous studies have indicated that binding of ERK to the DEF motifs of several substrates, including the Elk-1, SAP-1, and c-Fos transcription factors and the IEX-1 early response gene product, results in phosphorylation of specific (S/T)P sites positioned at DEF motif N termini and subsequent functional activation (15–17, 35). Protein sequence analysis showed that there are four SP sites located at the N terminus (Ser^{296} and Ser^{237}) and C terminus (Ser^{359} and Ser^{364}) of the MKP-1 DEF motif (Fig. 1). To...
determine whether they are involved in ERK phosphorylation following docking to the DEF motif, we generated the GST-tagged N-MKP-1 mutants S296A, S323A, S296A/S323A, S359A, S364A, and S359A/S364A, in which Ala was substituted for one or two Ser residues. Fig. 4 shows that the in vitro phosphorylation efficiency of active ERK2 declined markedly in S296A and S323A and that S296A/S323A was nearly unphosphorylated (first row), despite active ERK2 being bound to all these mutants (rows 2 and 3). Conversely, active ERK2 phosphorylated the S359A, S364A, and S359A/S364A mutants at similar levels compared with ΔN-MKP-1 (Fig. 4). We next generated an anti-phospho-MKP-1 antibody to provide further evidence for phosphate occupancy at Ser296. After nonradioactive in vitro ERK2 kinase assay, all mutants except S296A and S296A/S323A were recognized by the anti-phospho-Ser296 MKP-1 antibody (Fig. 4B). These results suggest that active ERK docking to the MKP-1 DEF motif leads to phosphorylation of its N-terminal Ser296/Ser323 sites and that the DEF motif C-terminal S359A/S364A mutations do not interfere with the ability of active ERK2 to phosphorylate DEF motif N-terminal SP sites.

To establish the involvement of the four SP sites in ubiquitin-mediated proteolysis of MKP-1 triggered by ERK signaling in vivo, we constructed the FLAG-tagged full-length MKP-1 mutants S296A, S323A, S296A/S323A, and S359A/S364A and expressed them together with HA-MKK1-CA or pcDNA3 in H293 cells. Unlike what occurred with WT MKP-1, forced expression of MKK1-CA in H293 cells with ALLN

**FIGURE 7.** ERK activation by serum decreases the half-life of endogenous MKP-1 via phosphorylation at Ser296, leading to ubiquitin-proteasome-mediated proteolysis. A, cells were starved for 2 days and co-treated with serum (10–20% (left panels) and 20% (middle panels)) and cycloheximide (CHX; 10 μg/ml) for 3 h (left panels) or for 15 min to 3 h (middle panels) before WCE isolation. Alternatively, the serum-starved cells were left untreated or pretreated with U0126 (5 μM) for 1 h before co-treatment with 20% serum and cycloheximide for 3 h (right panels). As indicated, ALLN (10 μM) was supplied for the final 1 h (left and right panels) or 10 min (middle panels) to reveal phosphorylated and ubiquitinated MKP-1. The proteins in the WCE were analyzed by Western blotting using the indicated antibodies. Immunoprecipitation (IP) was performed using anti-phospho-Ser296 MKP-1 (pMKP1(S296)) antibody, followed by immunoblotting (IB) using anti-ubiquitin (Ub) antibody. The results shown are representative of three experiments. IgH, immunoglobulin H chain. B, ERK suppression increased the half-life of endogenous MKP-1 upon serum stimulation in H293 cells. The serum-starved cells were pulse-labeled with [35S]methionine for 1 h in the presence or absence of U0126 (5 μM) before stimulation with 10% serum for 0–3 h in a chasing medium. The endogenous MKP-1 half-life was estimated by averaging three independent experiments.
MKP-1 Proteolysis via ERK and SCF<sup>Skp2</sup>

FIGURE 8. The Ser<sup>296</sup>/Ser<sup>323</sup> phosphodegron is essential for the association between MKP-1 and SCF<sup>Skp2</sup> in vivo upon MKK1/2-ERK signaling, which is not affected by the S359A/S364A mutations. Cells were transfected with HA-MKK1-CA, HA-MKK2-CA, or pcDNA3 (A) or with FLAG-tagged WT MKP-1, MKP-1(S296A/S323A), or MKP-1(S359A/S364A) along with HA-MKK1-CA or pcDNA3 (B). Cells were then incubated for 2 days and treated with 10 μM ALLN for the final 3 h. In C, serum-starved cells were cultured with 10–20% serum for 3 h in the presence of ALLN (10 μM) for the final 1 h. The association between MKP-1 and Skp2 was determined by immunoprecipitation (IP) using antibodies against Skp2, MKP-1, and FLAG, followed by Western blotting using the indicated antibodies. The proteins in the WCE were also subjected to Western blotting to monitor transfection efficiency and equal loading. The results shown are representative of four experiments. IgH and IgL, immunoglobulin heavy and light chains, respectively; p-MKP-1(S296), anti-phospho-Ser<sup>296</sup> MKP-1 antibody; endo., endogenous.

JANUARY 13, 2006 • VOLUME 281 • NUMBER 2

MKP-1 Proteolysis via ERK and SCF<sup>Skp2</sup> is the E3 Ligase for MKP-1 Ubiquitination and Proteolysis Triggered by ERK—ERK activity is vital in driving cell cycle progression, particularly at the G<sub>1</sub>/S transition (8). The E3 activity of SCF<sup>Skp2</sup> controls S phase entry and progression by recognizing the phosphodegrons of targeted proteins such as p27<sup>Kip1</sup>, leading to proteolysis (31, 32). We therefore examined the possibility that SCF<sup>Skp2</sup> is involved in ERK-triggered MKP-1 proteolysis via recognition of the phosphodegron. As shown in Fig. 8A, endogenous Skp2 coexisted with MKP-1 containing phospho-Ser<sup>296</sup> in an immunocomplex derived from H293 cells during forced expression of MKK1-CA or MKK2-CA; by contrast, such a complex was not observed in the absence of ERK signaling. The Skp2/MKP-1 immunocomplex also contained the other SCF subunits Skp1 and Cul1 and the accessory protein Csk1 (Fig. 8A).

Unlike FLAG-tagged MKP-1, and all mutants (Fig. 5, B, middle panel; and C, left panel). Conversely, forced expression of MKK1-CA in H293 cells caused S359A/S364A polyubiquitination and degradation at levels similar to those occurring in WT MKP-1 (Fig. 5). These results suggest that the Ser<sup>296</sup>/Ser<sup>323</sup> phosphoacceptor is involved in the MKP-1 polyubiquitination and proteolysis in vivo, which is not affected by the S359A/S364A mutations. Moreover, compared with the coexpressed control vector, the MKK1-CA-induced phospho-ERK levels were attenuated during coexpression of WT MKP-1 and all mutants (Fig. 5, B, second row; and C, right panel).

We next measured the degradation rate of FLAG-tagged full-length WT MKP-1 and mutants ANAP, S296A/S323A, and S359A/S364A during expression in H293 cells upon starvation and restimulation with 10% serum for 0–3 h by pulse-chase analysis. The half-lives of exogenous WT MKP-1 and mutants were ∼2–2.5 h in serum-starved cells (Fig. 6). Serum markedly decreased the half-lives (∼1 h) of WT MKP-1 and S359A/S364A, but did not affect the degradation rate of ANAP and S296A/S323A (Fig. 6). These results suggest that the DEF motif and the Ser<sup>296</sup> and Ser<sup>323</sup> residues are necessary for rapid destruction of MKP-1 in response to serum, which is not significantly affected by the S359A/S364A mutation.

The ability of ERK signaling to trigger MKP-1 phosphorylation, ubiquitination, and proteolysis was further established upon serum stimulation of quiescent cells entering the cell cycle. H293 cells were starved for 2 days and then co-treated with serum (10–20%) and cycloheximide for 15 min to 3 h in the presence or absence of ALLN. Fig. 7A shows that, in the absence of ALLN, serum-activated ERK was accompanied by dose-dependent (left panels) and time-dependent (middle panels) decreases in MKP-1 protein levels and that the serum-induced phospho-ERK preceded the increased phospho-Ser<sup>296</sup> MKP-1, in which polyubiquitination was also evident with ALLN. When the ERK activation was blocked by U0126, serum could not cause phospho-Ser<sup>296</sup> or polyubiquitination in MKP-1 in the presence of ALLN or lower the MKP-1 protein levels in the absence of ALLN (Fig. 7A, right panels). The phenomenon was also observed in similar experiments without addition of cycloheximide (data not shown). Moreover, the half-life of endogenous MKP-1 in H293 cells was estimated to be ∼2 h upon serum starvation and was notably reduced upon 10% serum stimulation (Fig. 7B). By contrast, inhibition of MKK1/2 by U0126 blocked the ability of serum to trigger MKP-1 proteolysis in H293 cells (Fig. 7B). These results indicate that, upon serum stimulation of quiescent cells, ERK activation directs MKP-1 phosphorylation at Ser<sup>296</sup> (termed a phosphodegron), leading to proteolysis via the ubiquitin-proteasome pathway.

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WT MKP-1, the S296A/S323A mutant failed to associate with Skp2 upon ERK signaling (Fig. 8B). Conversely, the S359A/S364A mutant exhibited phospho-Ser-296 and formed a complex with Skp2 upon ERK signaling (Fig. 8B). These results suggest that the Ser296/Ser323 phosphodegragon is essential for MKP-1 and SCFSkp2 association in vivo and that the S359A/S364A mutations do not interfere with formation of such a complex. Furthermore, although endogenous MKP-1 did not associate with SCFSkp2 in serum-starved cells, its phospho-Ser-296 form bound to SCFSkp2 during forced expression of MKK1-CA or serum stimulation (Fig. 8B and C). These results suggest that MKP-1 is a cellular substrate of the SCFSkp2 E3 ligase.

We next performed an *in vitro* assay to verify that the E3 activity of SCFSkp2 directs MKP-1 ubiquitination. After nonradioactive *in vitro* ERK2 kinase assay, the N-terminally truncated (Fig. 9A, *left panel*) or WT (right panel) MKP-1 could be polyubiquitinated as long as all components (Uba1 (E1), Ubch3 (ubiquitin carrier protein), the SCFSkp2-Cks1 complex (E3), and ubiquitin) were present in the reaction mixture. The SCFSkp2-Cks1-mediated *in vitro* ubiquitination was markedly suppressed when the ΔN-MKP-1 substrate had not been phosphorylated by active ERK2 (Fig. 9B). Also, the ΔN-MKP-1 (S296A/S323A) and ΔN-MKP-1 (ANAP) mutants, which were hardly phosphorylated by active ERK2 (Figs. 2 and 4), were not targets of SCFSkp2-Cks1 *in vitro* (Fig. 9B) (data not shown). In contrast, the SCFSkp2-Cks1 complex triggered ubiquitination in ΔN-MKP-1(S359A/S364A) after active ERK2 phosphorylation. These results strongly argue that the MKP-1 Ser296/Ser323 phosphodegragon generated by active ERK is essential for the SCFSkp2-Cks1-mediated ubiquitination *in vitro*, whereas the Ser359/Ser364 sites and N-terminal domain of MKP-1 are dispensable.

To determine whether Skp2 E3 activity participates in the ERK-directed ubiquitination and proteolysis of MKP-1 *in vivo*, we modulated Skp2 levels in H293 cells via forced expression of FLAG-Skp2 or knockdown of Skp2 using a specific siRNA duplex. Upon MKK1-ERK signaling, FLAG-Skp2 dose-dependently decreased the levels of endogenous MKP-1 and increased its polyubiquitination (Fig. 10). Conversely, depletion of endogenous Skp2 suppressed MKP-1 ubiquitination and proteolysis stimulated by forced expression of MKK1-ERK signaling (Fig. 10). The protein level of p27kip1 was also examined as a positive control for SCFSkp2 activity. Forced expression of FLAG-Skp2 decreased the p27kip1 level, whereas Skp2 depletion increased it; however, these events were ERK-independent (Fig. 10). Furthermore, pulse-chase analysis showed that knockdown of Skp2 decreased the degradation rate of endogenous MKP-1 stimulated by serum in H293 cells (Fig. 11A). These results indicate that SCFSkp2 is the E3 ligase mediated in ERK-directed MKP-1 proteolysis *in vivo*.

To further establish that the cooperation of ERK with SCFSkp2 for MKP-1 proteolysis can prolong the ERK signaling under physiological conditions, we measured the effects of Skp2 overexpression or knockdown on the kinetics of ERK activity and MKP-1 levels upon serum stimulation. As shown in Fig. 11 (B and C), expression of FLAG-tagged Skp2 significantly increased the serum-stimulated phospho-ERK level and prolonged its duration in H293 cells, in which endogenous MKP-1 degradation was markedly accelerated; conversely, depletion of endogenous Skp2 significantly decreased the phospho-ERK level, and MKP-1 degradation was suppressed. These results suggest that, upon serum stimulation, sustained ERK activity can be achieved via MKP-1 proteolysis triggered by a positive feedback mechanism in cooperation with SCFSkp2.

**DISCUSSION**

**The Two ERK-docking Sites in MKP-1 Display Distinct Functions**

MKP-1 phosphatase activity is enhanced upon ERK2 association, which requires the RRR motif (residues 53–55) in the MKP-1 KIM domain and Asp319 in the ERK2 common docking domain (25). This common docking/KIM docking-coupled phosphatase activation plays an important role in feedback down-regulation of ERK signaling. By contrast, as we reported previously (30), ERK activation upon Pb(II) exposure or MKK1/2-CA overexpression triggers MKP-1 degradation via the ubiquitin-proteasome pathway. Here, we have shown that ERK-directed MKP-1 phosphorylation, ubiquitination, and proteolysis also occur during serum stimulation of quiescent cells. Using several N-terminally
truncated KIM domain mutants, we found that, in vitro, the MKP-1 C-terminal DEF motif serves as a docking site for active ERK, leading to phosphorylation of Ser296 and possibly Ser323. The DEF motif and Ser296/Ser323 are absolutely required for ERK-directed ubiquitination in WT MKP-1, leading to proteolysis in vivo. ERK also triggers ubiquitination in /H9004 N-MKP-1, suggesting that the N-terminal module is not essential for ubiquitin-mediated MKP-1 destruction. Intriguingly, the MKK1-CA-induced phospho-ERK level was attenuated by coexpression with WT MKP-1 or any of the full-length MKP-1 mutants constructed here (Figs. 3C and 5C, right panels), suggesting that the exogenous WT MKP-1 phosphatase may down-regulate ERK activity and that these MKP-1 mutants may sustain the phosphatase activity. These findings are consistent with the observation that an MKP-1 mutant missing C-terminal residues 315–367 retains phosphatase activity (21, 26). Thus, the MKP-1 C-terminal module is probably dispensable for phosphatase activation. Taken together, the foregoing implies that ERK performs dual functions in regulating MKP-1 through docking to its KIM domain for phosphatase activation and to its DEF motif for MKP-1 destruction.

It has been reported that the full-length MKP-1 mutants S359A, S364A, and S359A/S364A are poor substrates for active ERK2 (28). Although there was no evidence for phosphate occupancy at Ser359 and Ser364, it has been suggested that they are the major sites for ERK phosphorylation, causing a decrease in MKP-1 proteolysis (28). However, we found that, in the N-terminally truncated form of MKP-1 (residues 1–59), the S359A and/or S364A mutant was phosphorylated as efficiently as ΔN-MKP-1 by active ERK (Fig. 4). We also confirmed that active ERK2 indeed phosphorylated the full-length MKP-1 mutants S359A and/or S364A to a significantly lesser degree than it phosphorylated WT MKP-1; however, these full-length mutants did not perturb Ser296 phosphorylation by ERK in vitro (data not shown). That the N-terminal module is required for the decreased extent of phosphorylation of the MKP-1 mutants S359A and/or S364A by active ERK2 suggests that docking to the KIM domain may direct Ser359/Ser364 phosphorylation. Such a hypothesis deserves further investigation. Nevertheless, the results shown here indicate that the S359A/S364A mutant behaves just like WT MKP-1 in being phosphorylated at the Ser359/Ser364 domain by active ERK2, leading to ubiquitination and destruction.

On the other hand, although forced activation of ERK signaling did not cause ubiquitination in the MKP-1 mutants ANAP, S296A, S323A, and S296A/S323A, it did cause a decrease of ~20% in each of these mutants (Figs. 3C and 5C, right panels). These results suggest that ERK may also trigger MKP-1 destruction through a pathway independent of...
ubiquitination and/or degrons. This hypothesis warrants further exploration.

MKP-1 Is a Sensor That Active ERK Uses to Control Its Signaling Duration—Depending on the cell type and stimulus, the strength and duration of ERK activation regulate many aspects of cell fate, including the control of S phase entry (7, 8). For example, transient ERK signaling is not permitted past the late G1 phase in fibroblasts, whereas sustained ERK signaling as stimulated by platelet-derived growth factor is necessary for transcriptional activation of genes, including cyclin D, to drive S phase entry (36). ERK signaling also induces p21<sup>Cip1</sup> levels in early G1 phase upon growth factor stimulation, which promotes the assembly of cyclin D-CDK4/6 (37). However, the p21<sup>Cip1</sup> level induced by strong ERK signaling can inhibit CDK2 activity, causing G1 phase arrest (38).

Thus, initial ERK signaling stimulation must exceed a high threshold followed by timely conversion to a sustained and moderate level for mitogenic promotion of S phase entry. However, it is unclear at present how sustained ERK signaling can be established when cells encounter robust extracellular stimuli.

Several transcription factors have been shown to differentially sense ERK signaling duration (17, 18). ERK activation rapidly increases transcription of IEGs such as c-fos via phosphorylation and activation of promoter-bound transcription factors such as Elk-1 (39). The newly synthesized IEG proteins such as c-Fos, Fra-1, Fra-2, and c-Myc are, however, unstable upon transient ERK activation. Continued expression of these IEG proteins and execution of their biological functions require sustained ERK activation for docking to the DEF motif and

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FIGURE 11. Skp2 modulates the kinetics of MKP-1 degradation and sustained ERK activity upon serum stimulation. A, Skp2 depletion increased the half-life of endogenous MKP-1 in serum-stimulated cells. Cells were transfected with siRNA duplexes (200 nM each) specific to Skp2 (si-Skp2) or lamin (si-Lamin), cultured in complete medium for 24 h, and starved in 0.1% serum for another 24 h. The cells were then pulse-labeled with <sup>35</sup>S]methionine for 1 h prior to stimulation with 10% serum for 0–3 h in a chasing medium. The endogenous MKP-1 proteins were immunoprecipitated using anti-MKP-1 antibody and then subjected to gel electrophoresis, followed by autoradiography. The half-life of endogenous MKP-1 was estimated by averaging three independent experiments. B, cells that had been transfected with the indicated vectors (4 µg each) or siRNA (200 nM each) were subjected to serum starvation for 1 day before stimulation with 5% serum for 0–3 h. The proteins in the WCE were analyzed by Western blotting using the indicated antibodies. C, shown are the results from quantitative analysis of the relative MKP-1 and phospho-ERK (p-ERK) levels obtained in B. The results were calculated by averaging three independent experiments.
targeting site-specific phosphorylation (17, 18). Although thought to act as molecular sensors of ERK signaling duration, these transcription factors do not explain how sustained ERK activation is achieved.

The finding that ERK exhibits dual functions in regulating MKP-1 through docking to its KIM and DEF sites allows us to propose a model for elucidating how ERK auto-controls its signaling duration. Because MKP-1 is also an IEG protein rapidly induced in response to ERK activation (34), the dynamics of ERK activation should be determined by the stoichiometric ratio of active ERK to MKP-1. It is worth noting that unactive ERK2 is able to bind to the MKP-1 KIM domain (25), whereas only in its active form can ERK2 dock to the MKP-1 DEF motif (Fig. 2). Upon weak stimulation, ERK increases MKP-1 transcription and phosphatase activity to feedback-dephosphorylate the kinase, resulting in low stoichiometric ratios of active ERK to MKP-1 and net transient signaling. When active ERK overwhelms MKP-1 in the presence of strong mitogenic stimuli, active ERK docks to the MKP-1 DEF motif in the C-terminal module and triggers MKP-1 destruction (Fig. 2); meanwhile, active ERK also induces MKP-1 resynthesis, and a balanced ratio of active ERK to MKP-1 over the threshold is set, resulting in net sustained signaling. Once the DEF module in MKP-1 is triggered, active ERK may predispose docking to IEG DEF sites, thereby prolonging the transcriptional activities. The DEF module in MKP-1 may thus serve as a sensor for transmitting sustained ERK signaling to the IEG transcription factors that act as effectors to promote cell cycle progression.

**SCFSkp2 Is the E3 Ligase for ERK-directed MKP-1 Ubiquitination**—We have further shown here that SCFSkp2 is the principal E3 ligase for ERK-directed MKP-1 ubiquitination and proteolysis in vivo and in vitro. First, upon ERK signaling, endogenous phospho-Ser296 MKP-1 physically interacts with the SCFSkp2 complex and its accessory protein Cks1, whereas ERK activation does not promote SCFSkp2 association with the MKP-1 mutant S296A/S323A in vivo. Second, the Ser296/Ser323 phosphodegron generated via active ERK docking to the MKP-1 DEF motif is absolutely required for SCFSkp2-mediated ubiquitination in vitro. Third, exogenous Skp2 expression dose-dependently triggers MKP-1 ubiquitination and proteolysis; again, ERK signaling is absolutely required. By contrast, endogenous Skp2 depletion stabilizes MKP-1 upon ERK activation. Moreover, the kinetic results shown here indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells. Taken together, these results indicate that the ERK-directed MKP-1 proteolysis requires SCFSkp2 E3 activity, which can prolong the duration of ERK signaling upon serum stimulation of resting cells.
MKP-1 Proteolysis via ERK and SCF^Kip2^

gron generated by the kinase activity of cyclin E/A-CDK2 (p27^Kip1^), cyclin E-CDK2 (p27^Kip2^), or cyclin D-CDK4/6 (p130). Our finding that phosphorylation of MKP-1 at Ser^{296} is essential for SCF^Kip2^ association and ubiquitination is consistent with the notion that a phosphodegron is necessary for Skp2 recognition via its C-terminal leucine-rich repeat (31, 32). Moreover, the phosphodegron in MKP-1 achieved by ERK activity represents a novel mechanism different from that by which CDK activity generates all known SCF^Kip2^ phosphodegrons. We have also demonstrated that Skp2-triggered p27^Kip1^ ubiquitination is ERK-independent (Fig. 10), which supports selectivity being achieved by specific docking interactions between distinct kinases and substrates.

Conclusion—We have reported here the MKP-1 DEF motif is essential for ERK docking to generate the Ser^{296}/Ser^{321} phosphodegron that targets MKP-1 for proteolysis via ERK and SCF^Kip2^. This finding is consistent with the observation that Skp2 accelerates the abilities of either activated N-Ras or H-Ras (both ERK upstream activators) to induce cell transformation and tumors (51, 52).

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