MEKK3 interacts with the PA28 γ regulatory subunit of the proteasome

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The proteasome is a multisubunit proteolytic enzyme comprising activator complexes bound to the 20 S catalytic core. The functions of the proteasomal activator (PA) 700 in ubiquitin/ATP-dependent protein degradation and of the PA28 α/β activators in antigen presentation are well defined. However, the function of a third PA, PA28 γ , remains elusive. We now show that mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) kinase kinase 3 (MEKK3), a MAPK kinase kinase (MAPKKK) involved in MAPK kinase 7 (MKK7)–c-Jun N-terminal kinase ('JNK') and MKK6–p38 signalling, can bind PA28 γ but not PA28 α . In contrast, B-Raf, a MAPKKK specific for the MAPK/ERK kinase ('MEK')–ERK module, binds PA28 γ and α . The PA28 γ -binding domain of MEKK3 is located within

its N-terminal regulatory domain (amino acids 1–178). Expression of MEKK3 in Cos-7 cells led to an increase in endogenous and co-expressed PA28 γ protein levels, whereas kinase-deficient MEKK3 had no effect on PA28 γ expression. Furthermore, *in vitro* assays indicated that PA28 γ was a MEKK3 substrate. MEKK3 represents the first protein kinase capable of binding and phosphorylating a PA, and provides a potential mechanism to link stress-activated protein kinase signalling with the PA28 γ -dependent proteasome.

Key words: mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) kinase kinase 3 (MEKK3), proteasomal activator, stress signalling.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascades are highly conserved in structure and organization and mediate a variety of cellular responses to stress and extracellular stimuli [1]. Each cascade consists of a module of sequentially acting cytoplasmic kinases that includes a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and the MAPK itself. MAPK family members include the extracellular-signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 kinases. Once activated, MAPKs exert their effects by phosphorylating cytoplasmic proteins and by translocating to the nucleus where they regulate transcription factors that control gene expression [1].

MAPK/ERK kinase kinase 3 (MEKK3) belongs to a family of serine/threonine kinases that act as MAPKKKs in the JNK and p38 activation pathways that are induced by cellular stress (e.g. UV and γ radiation, osmotic stress and heat shock) and inflammatory cytokines (e.g. tumour necrosis factor- α and interleukin-1) [2]. Stress-activated protein kinases have been implicated in various cellular responses to these stimuli, including apoptosis, oncogenic transformation and inflammation. Although the substrate specificities of MEKK3 have been defined [3,4] and are consistent with its involvement in JNK and p38 activation, the signalling events controlling MEKK3 are poorly understood. In order to identify novel upstream regulators and downstream substrates of MEKK3, the full-length protein kinase was used to screen a yeast two-hybrid phaeochromocytoma cell (PC12) cDNA library. In addition to isolating a variety of 14-3-3 family members

and three unknown clones encoding incomplete proteins, a proteasomal activator (PA) subunit, PA28 γ , was identified as a new MEKK3-interacting partner.

The proteasome is a large multisubunit proteolytic enzyme complex that is implicated in a variety of cellular processes, including antigen presentation, cell-cycle progression and the control of gene expression [5,6]. The catalytic activity of the proteasome is intrinsic to the 20 S core complex, which possesses a barrel-like structure composed of two outer α -rings and two inner β -rings [5,6]. Catalytic activation and substrate specificity of the 20 S proteasome is dependent on association of activator complexes with both ends of the core complex. Binding of PA700, itself a multimeric complex, forms the 26 S proteasome and directs degradation of polyubiquitinated proteins in an ATP-dependent manner [5,6]. Alternatively, exchange of certain 20 S subunits and binding of PA28, a heptameric complex formed of homologous α and β subunits, allows proteasomal degradation of small peptides independently of ubiquitin conjugation or ATP hydrolysis [7]. Proteasomal complexes are distributed throughout the cytoplasm and the nucleus [8], and PA28–20 S is also located at the endoplasmic reticulum [9]. Interferon- γ (IFN- γ) is a key mediator of the immune response and stimulates the expression of PA28 α and β subunits, thereby enhancing generation of peptides by the PA28–20 S proteasome for antigen presentation by MHC class I receptors [10,11]. PA700–20 S and PA28–20 S proteasomes have been proposed to sequentially degrade polyubiquitinated proteins into small peptides [9,12]. However, hybrid proteasomes containing a PA700 and PA28 complex at either end of the 20 S catalytic core have also been identified

Abbreviations used: CMV, cytomegalovirus; DPBS, Dulbecco's Ca/Mg-free PBS; ERK, extracellular-signal-regulated kinase; Flag, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; GST, glutathione S-transferase; HA, haemagglutinin; IFN- γ , interferon- γ ; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEKK, MAPK/ERK kinase kinase; MKK, MAPK kinase; JNK, c-Jun N-terminal kinase; PA, proteasomal activator; MEKK, MAPK/ERK kinase kinase; KD, kinase-dead.

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[13–15]. These PA700–20 S–PA28 proteasomes are found in the cytoplasm and in association with the endoplasmic reticulum, and may contribute to ATP-dependent generation of peptides from intact proteins [9].

PA28 α and β subunits show 48 % amino acid sequence identity [16]. A third homologue, PA28 γ , is located in the nucleus and is unlikely to contribute to antigen generation for MHC-I receptors. It was first discovered as Ki autoantigen in patients with systemic lupus erythematosus [17]. PA28 γ shares 34 % and 39 % homology with PA28 α and β respectively, and forms a homoheptameric complex that stimulates the trypsin-like activity of the 20 S proteasome [16]. Whereas IFN- γ increases expression and association of PA28 α and β with the 20 S complex for antigen presentation, PA28 γ is not induced by IFN- γ [7,18]. Although the function of PA28 γ remains elusive, PA28 γ -depletion experiments in mice and embryonic fibroblasts suggest that it may be involved in cell-cycle control [19]. In addition, PA28 α , β and γ appear to exist as phosphoproteins in the cell, suggesting that phosphorylation may also contribute to regulation of the proteasome, although the kinase responsible for phosphorylating these subunits has not been identified [20].

In the present study, we report that MEKK3, a MAPKKK that specifically activates the MKK7–JNK and MKK6–p38 stress kinase modules, directly interacts with and phosphorylates PA28 γ . The kinase activity of MEKK3 also increases PA28 γ protein levels in intact cells, most likely by increasing PA28 γ stability. Collectively, these observations suggest a novel mechanism for regulation of the PA28 γ –20 S proteasome by a stress kinase signalling pathway.

EXPERIMENTAL

Plasmids

The generation of the PC12 two-hybrid cDNA library and cloning of pPC97-c-Raf-1, pPC97-A-Raf and pPC97-B-Raf have been reported previously [21,22]. The two-hybrid vectors pPC86 (prey) and pPC97 (bait) [23] were gifts from D. Nathans. A murine brain cDNA fragment containing the entire coding sequence of MEKK3 was amplified by PCR from Clone 11 [24] in pBluescript and inserted into *SalI/NotI* of pPC97. For generation of the MEKK3 (1–178) truncation in pPC97, a Klenow reaction was performed on *NotI*-digested pPC97-MEKK3, followed by *SmaI* digestion and religation of the vector fragment. pPC97-MEKK3 (128–626) was generated by a *SalI/BclI* digestion, end-filling of both sites, and vector religation. The kinase domain and N-terminal regulatory region of MEKK3 were separately amplified by PCR from Clone 11 and inserted into *SalI/NotI* of pPC97 to produce pPC97-MEKK3 (367–626) and pPC97-MEKK3 (1–366) respectively. The generation of haemagglutinin (HA)-tagged, Flag-tagged (where Flag is Asp-Tyr-Lys-Asp-Asp-Asp-Lys) and untagged MEKK3 in pCMV5 (where CMV is cytomegalovirus) for mammalian expression has been described previously [3,24]. Kinase-dead (KD) HA-MEKK3 in pCMV5 was generated by replacing the active-site Lys³⁹¹ with methionine using the Quickchange mutagenesis system (Stratagene). pCMV5-MEKK3 (1–178) was generated by PCR cloning from Clone 11 into *EcoRI/SalI* of pCMV5. The N-terminal regulatory half of MEKK3 was amplified by PCR from Clone 11 and digested with *EcoRI* and *SalI* to produce two fragments due to an internal *EcoRI* site. The *EcoRI/SalI* fragment was ligated into the corresponding sites of pCMV5. This construct was then digested with *EcoRI* and the *EcoRI/EcoRI* fragment of the PCR-digest was inserted, thereby generating pCMV5-MEKK3 (1–366). Glutathione S-transferase (GST)–

MEKK3 (1–302) and GST–MEKK3 (302–626) were expressed from pGEX-4T-3 (Pharmacia) and prepared by subcloning of PCR-generated fragments containing a 5' *SalI* site and 3' *NotI* site [3]. Mouse PA28 γ cDNA was a gift from K. Tanaka [25]. The entire coding region of PA28 γ was amplified by PCR and subcloned into *XhoI/SalI* of pEGFP-C1 (Clontech). A similar fragment of PA28 γ containing a *BamHI* and *XhoI* site was generated by PCR and subcloned into pXJ40-HA (a gift from E. Manser) and pCMV5-Tag2B (Stratagene) for mammalian expression of HA–PA28 γ and Flag–PA28 γ respectively. pXJ40-HA-PA28 γ was digested with *BamHI* and *XhoI* and the isolated PA28 γ fragment was subcloned into *SmaI/XhoI* of pGEX-4T-3 with the *BamHI* site blunted. Bacterial expression vectors encoding GST–c-Jun, GST–JNK1 and GST–MKK7 were gifts from R. Davis and have been described previously [4]. Pfu Turbo *Taq* polymerase (Stratagene) was used for PCR. All other enzymes used for cloning were purchased from MBI-Fermentas. All plasmids were verified by restriction analysis and DNA sequencing. The sequence of all primers used for PCR will be made available upon request.

Yeast two-hybrid library screening

Yeast cultures were grown at 30 °C under standard liquid or solid culture conditions using YPD [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] or minimal SD media. The yeast strain HF7c [26] was sequentially transformed with the bait plasmid followed by the cDNA library. Transformants were grown on SD medium lacking the amino acids leucine, tryptophan and histidine. After 3–7 days, growing clones were tested for activation of the *lacZ* reporter gene in a β -galactosidase filter assay [21]. Positive clones were verified further by retransforming the isolated library plasmid into HF7c together with different bait plasmids. Clones shown to be positive for the β -galactosidase assay only in the presence of MEKK3 as bait were taken forward for sequence analysis.

Cell culture and transfection

Cos-7 cells were maintained and transfected as described previously [3].

Cell lysis, co-immunoprecipitation and Western-blot analysis

Cells were solubilized on ice with lysis buffer containing 50 mM Tris/HCl (pH 7.4) 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.5 % Nonidet P40, 1 mM Na₃VO₄, 1 mM PMSF and 10 μ g/ml leupeptin. Insoluble material was removed from cell lysates by centrifugation at 14 000 g for 10 min at 4 °C. An equal volume of Laemmli sample buffer was added and samples were boiled for 5 min. Proteins were resolved by SDS/PAGE [12 % (w/v) polyacrylamide gels] [27] and transferred on to a nitrocellulose membrane for 30 min at 12 V in transfer buffer [48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20 % (v/v) methanol] using a semi-dry transfer apparatus (Bio-Rad). Immunodetection of proteins was performed using the enhanced chemiluminescence reagents (Amersham Biosciences) as described previously [3].

For co-immunoprecipitation experiments, Cos-7 cells expressing Flag–MEKK3 from pCMV5 were lysed in 10 mM Tris/HCl (pH 7.4) containing protease inhibitors as described above. Each lysate (500 μ l) was cleared by centrifugation and incubated with 1 μ g of anti-Flag antibody (Sigma) for 1.5 h on ice. Glutathione–Sepharose (70 μ l of a 15 % slurry) was added and incubated with the cell extract overnight at 4 °C. The samples were then

spun for 10 s at 14 000 g, the supernatant was removed and the pellet washed six times with ice-cold 10 mM Tris/HCl (pH 7.4). Laemmli sample buffer (50 μ l) was added to the washed beads, which were then boiled for 5 min. Proteins were resolved by SDS/PAGE and analysed by Western blotting.

Antisera

The following antisera were used for Western blotting at the indicated dilutions: rabbit antiserum B130 (1:5000), raised against a purified recombinant GST-MEKK3 (1–301) [3]; rabbit antiserum against PA28 γ (1:500), a gift from K. Tanaka [25]; rabbit antibody to the HA epitope (1:1000) (Santa Cruz Biotechnology); and mouse monoclonal anti-(γ -tubulin) antibody (1:5000), raised to a synthetic γ -tubulin peptide (Sigma).

Protein purification

GST fusion proteins were purified by affinity chromatography [28] on glutathione–Sepharose (Pharmacia). Purified proteins were resolved by SDS/PAGE using 10 % (w/v) polyacrylamide gels in the presence of 0.1 % SDS [27] and quantified by comparative Coomassie Blue staining or by a Bradford protein assay (Sigma) using BSA as a standard. Purified PA28 γ was dialysed overnight at 4 °C against 10 mM Tris/HCl (pH 7.4) and concentrated using an Amicon YM-10 Centriprep filter device (Millipore).

GST-pulldown assays

Cos-7 cell lysates (200 μ l) expressing epitope-tagged proteins prepared in 10 mM Tris/HCl (pH 7.4), as described above, were incubated with purified glutathione–Sepharose bead-bound GST fusion proteins overnight at 4 °C with mixing. Beads were washed in 10 mM Tris/HCl (pH 7.4) and analysed for associated proteins by Western blotting as described for co-immunoprecipitation experiments.

Protein kinase assays

Immune complex protein kinase assays were performed in a final volume of 40 μ l of kinase buffer [10 mM Tris/HCl (pH 7.4), 20 mM MgCl₂ and 20 μ M ATP]. Recombinant MEKK3 protein (1 μ g) [Baculovirus/Sf9-expressed and purified MEKK3 (11–626) was generously given by N. Mody and P. Cohen] was preincubated in kinase buffer at 30 °C for 30 min to decrease background autophosphorylation. Protein kinase reactions were initiated by the addition of the appropriate recombinant substrate proteins and 5 μ Ci of [γ -³²P]ATP. Reactions were performed for 1 h at 30 °C and terminated by the addition of an equal volume of Laemmli sample buffer and boiling. Phosphorylated proteins were separated by SDS/PAGE and visualized by autoradiography.

Immunofluorescence

Cos-7 cells were grown either on sterile glass coverslips or in 6-well plates (Nunc) and, where indicated, the cells were co-transfected with 1 μ g of Flag-MEKK3 and 1 μ g of GFP-PA28 γ using Fugene-6 (Roche) according to the manufacturer's protocol. After 24 h, one set of transfected cells was treated with nocodazole (1 μ g/ml) for 12 h. The mitotic cells were collected by shake-off and attached to poly(L-lysine)-coated coverslips. The mitotic cells and the non-treated cells were then washed three times in Dulbecco's Ca/Mg-free PBS (DPBS) and fixed in

ice-cold (–20 °C) methanol for 30 min. Cells were rinsed five times with DPBS and blocked with 1 % (w/v) BSA (in DPBS) for 45–60 min at room temperature. A monoclonal anti-Flag M2 antibody (1:5000 dilution; Sigma) in DPBS containing 1 % (w/v) BSA was used to detect MEKK3. After incubation at room temperature for 60 min, the cells were washed five times with DPBS. Cells were then incubated for 60 min with a Texas Red-conjugated sheep anti-(mouse IgG) secondary antibody (1:100 dilution; Amersham Biosciences) in DPBS containing 1 % (w/v) BSA to visualize PA28 γ . The cells were washed five times with DPBS and stained with Hoechst 33258 (1 μ g/ml in DPBS) for 5 min at room temperature to label the nuclei. Following three washes with DPBS, the coverslips were mounted in 80 % (v/v) glycerol containing 3 % (w/v) n-propyl gallate and viewed using an inverted fluorescence microscope (Nikon TE300). Images were collected using an Orca ER digital CCD camera (Hamamatsu) and analysed using Openlab software (Improvision) and Photoshop 4 (Adobe).

RESULTS

Isolation of PA28 γ in a two-hybrid screen with MEKK3 as bait

MEKK3 has been shown to activate p38 and JNK by phosphorylating MAPK kinase 6 (MKK6) and MKK7 [4]. However, the signalling mechanisms involved in MEKK3 activation remain to be defined. In order to identify regulators and alternative substrates of MEKK3, we screened 10.5×10^6 clones of a PC12 two-hybrid cDNA library using full-length MEKK3 as bait. Of the 70 clones isolated, 55 scored positive based on the fact that the histidine and β -galactosidase phenotype of the yeast transformants was dependent on co-expression of MEKK3. The nucleotide sequences of these positive clones were obtained by automated sequencing and identified by a National Center for Biotechnology Information ('NCBI') Blast nucleotide database search. MEKK3 has been reported previously [29] to bind 14-3-3 proteins, and the majority of the clones isolated in the present screen represented members of the 14-3-3 protein family, with 14-3-3 ϵ being the most abundant (33 clones) (Table 1). In addition, seven 14-3-3 β , five 14-3-3 θ , three 14-3-3 ζ , one 14-3-3 γ and one 14-3-3 η clones were isolated (Table 1). Three clones predicted proteins with no significant sequence similarity to those in the database and are likely to represent novel proteins of unknown function (results not shown). Two clones corresponded to PA28 γ (Table 1), a nuclear homologue of cytoplasmic PA28 α and β that are involved in antigen presentation. Rat PA28 γ has not been cloned previously, but mouse PA28 γ cDNA encodes a protein of 254 amino acids [30]. The two independent rat PA28 γ clones isolated from the PC12 cDNA two-hybrid library predicted proteins that were identical with mouse PA28 γ but incomplete, lacking the first 78 and 106 amino acids. Because of this sequence identity, mouse PA28 γ cDNA [25] was used for all experiments, except for the direct yeast two-hybrid tests, to map the PA28 γ interaction domain of MEKK3, where the larger of the rat PA28 γ clones was used.

PA28 γ binds to the N-terminal regulatory domain of MEKK3

MEKK3 is a functional homologue of Raf family kinases that show absolute specificity for the MEK–ERK module. Interestingly, B-Raf was recently shown to bind PA28 α in two-hybrid studies [22]. To extend these observations, we assessed the ability of c-Raf-1, A-Raf and B-Raf to interact with PA28 γ . Of the three Raf kinases, only B-Raf showed detectable PA28 γ

Table 1 MEKK3 two-hybrid screen

A PC12 two-hybrid cDNA library (8.5×10^6 inserts) was screened with MEKK3 as a bait. Of 168 histidine positive (His^+) clones, 70 developed a blue colour in a β -galactosidase (LacZ) filter assay. Of these, 55 were true positives, based on their growth only in the presence of MEKK3, and identified by sequencing.

	Number of clones
Clones screened	10.5×10^6 (124 %)
His^+	168
LacZ^+	70
Clones identified	
14-3-3 β	7
14-3-3 ϵ	33
14-3-3 γ	1
14-3-3 θ	5
14-3-3 η	1
14-3-3 ζ	3
Unknown	3
PA28 γ	2

binding (Figure 1A). In addition, B-Raf also interacted with PA28 α (Figure 1B), confirming previous observations [22]. In contrast, MEKK3 did not associate with PA28 α in direct two-hybrid tests (Figure 1B), indicating that MEKK3 selectively interacts with PA28 γ . By using a range of MEKK3-deletion constructs, the PA28 γ -binding domain was localized to the first 178 amino acids of MEKK3 (Figure 1A). In direct two-hybrid tests, PA28 γ interacted with full-length MEKK3, its N-terminal regulatory domain [MEKK3 (1–366)] and a further truncation representing the first 178 amino acids of the enzyme [MEKK3 (1–178)]. No PA28 γ binding was detected to the kinase domain of MEKK3 [MEKK3 (367–626)] or to MEKK3 in which the N-terminal 127 amino acids were deleted [MEKK3 (128–626)] (Figure 1A). Interestingly, the blue colour in the β -galactosidase filter assay was stronger with MEKK3 (1–366) than with MEKK3 (1–178) or full-length MEKK3 (Figure 1A). This may indicate different affinities of interaction and suggests that the MEKK3 kinase domain may interfere with PA28 γ binding. In addition, although amino acids 1–178 of MEKK3 were sufficient for PA28 γ binding, additional enhancing interaction sites are likely to be present in MEKK3 (1–366). Thus MEKK3 interacts through its N-terminal regulatory domain with PA28 γ but not with PA28 α .

PA28 γ interacts with MEKK3 in GST-pulldown assays and co-immunoprecipitation experiments

To confirm the results of the direct two-hybrid tests, HA epitope-tagged PA28 γ was transiently expressed in Cos-7 cells and the derived cell lysates applied to GST–MEKK3 fusion proteins precoupled to glutathione–Sepharose beads. The GST fusion protein containing the N-terminal regulatory domain of MEKK3 [GST–MEKK3 (1–301)] efficiently precipitated HA–PA28 γ from Cos-7 cell lysates, whereas free GST and GST fused to the kinase domain of MEKK3 [GST–MEKK3 (302–626)] did not (Figure 2A). Reciprocal GST-pulldown experiments were also performed in which GST–PA28 γ was used as a ligand to affinity precipitate HA–MEKK3 expressed in Cos-7 cells. GST–PA28 γ precipitated HA–MEKK3, whereas free GST did not (Figure 2B). To determine whether MEKK3 and PA28 γ could also interact in cells, Flag–MEKK3 was co-expressed with empty vector or HA–PA28 γ in Cos-7 cells. MEKK3 was immunoprecipitated using an antibody to the Flag epitope, and PA28 γ was detected in the precipitates by Western-blot

Figure 1 Interaction of MEKK3 with PA28 γ

Yeast strain HF7c was co-transformed with bait and prey constructs as indicated. Growth on medium lacking histidine (–His) and the development of blue colour in a β -galactosidase filter assay (LacZ) indicate interaction. The closed boxes represent regions of sequence similarity. (A) PA28 γ does not bind to c-Raf-1 or A-Raf, but interacts with B-Raf and full-length MEKK3. The N-terminal regulatory half and the N-terminal 178 amino acids of MEKK3, but not the kinase domain of MEKK3 or a construct lacking the first 127 amino acids, show association with PA28 γ . (B) Interaction of MEKK3 with PA28 γ is specific, since it does not bind to PA28 α . B-Raf was shown previously [22] to associate with both PA28 α and PA28 γ .

analysis using an anti-HA antibody. PA28 γ co-precipitated with MEKK3 (Figure 3), confirming that these proteins can interact in mammalian cells.

MEKK3 enhances PA28 γ protein expression in Cos-7 cells

Western blotting of proteins from co-transfection studies suggested that MEKK3 enhanced PA28 γ expression (Figure 3, third panel). To examine this effect further, HA–MEKK3 and HA–PA28 γ were expressed in Cos-7 cells either alone or in combination and detected by Western blotting using an anti-HA antibody. PA28 γ protein expression was greatly increased by co-expression with MEKK3 (Figure 4A). Titration of the transfected MEKK3 plasmid to vary MEKK3 expression showed a concentration-dependent effect on PA28 γ protein levels (Figure 4A). Control immunoblots showed that the expression of γ -tubulin was uniform in all cell lysates (Figure 4A). Since HA–PA28 γ expression from pCMV5 is driven from a powerful CMV

Figure 2 GST-pulldown assays of MEKK3 and PA28 γ association

Cos-7 cells transfected with empty vector (pCMV5), HA-PA28 γ or HA-MEKK3 in pCMV5 were lysed and incubated with equivalent amounts of the appropriate GST fusion protein coupled to glutathione-Sepharose beads, as indicated. Association of HA-PA28 γ and HA-MEKK3 were detected by Western blotting using an anti-HA antibody. The first two lanes represent HA protein expression in total cell lysates. (A) PA28 γ is precipitated by the N-terminal regulatory half of MEKK3 [GST-MEKK3 (1–301)], but not by the kinase domain [GST-MEKK3 (302–626)]. (B) GST-PA28 γ pulls down full-length MEKK3.

promoter, it is unlikely that MEKK3 increases the transcription of PA28 γ . Indeed, MEKK3 expression does not affect the expression of a variety of other proteins from pCMV5 (results not shown), confirming that MEKK3 does not regulate the CMV promoter. Furthermore, overexpression of HA- or Flag-MEKK3 alone also increased native PA28 γ protein levels in Cos-7 cells (Figure 4B). To determine whether MEKK3-induced PA28 γ expression was dependent on a physical interaction or on MEKK3 kinase activity, the N-terminal 178 amino acids of MEKK3 were expressed in Cos-7 cells together with PA28 γ . This region of MEKK3 lacks kinase activity, but is capable of interacting with PA28 γ in two-hybrid tests (Figure 1C). Expression of MEKK3 (1–178) had no effect on the level of co-expressed PA28 γ and did not alter the ability of full-length MEKK3 to increase PA28 γ expression (Figure 4C). Thus the interaction domain of MEKK3 alone is insufficient to elevate PA28 γ protein levels, presumably because it lacks catalytic activity. To test this possibility, PA28 γ was expressed alone or in combination with wild-type or KD MEKK3 (MEKK3 KD). Whereas catalytically active MEKK3 increased PA28 γ expression, comparable expression of MEKK3 KD did not (Figure 4D). Thus the kinase activity of MEKK3 is necessary to enhance PA28 γ expression in Cos-7 cells.

Figure 3 PA28 γ and MEKK3 interact in co-immunoprecipitation experiments

Cos-7 cells were transfected with plasmids (2 μ g each) encoding Flag-MEKK3 and HA-PA28 γ either alone or in combination, as indicated. Empty pCMV5 vector (2 μ g) was used to make the total amount of transfected DNA equivalent. MEKK3 in cell lysates was immunoprecipitated (IP) using an anti-Flag antibody and detected using B130 antibody; PA28 γ was detected using anti-HA antibody. WB, Western blot.

MEKK3 phosphorylates PA28 γ *in vitro*

Because enhanced expression of PA28 γ was dependent on the catalytic activity of MEKK3, we tested whether PA28 γ was phosphorylated by MEKK3. GST-PA28 γ was used as a substrate for *in vitro* kinase assays with recombinant MEKK3 purified from baculovirus-infected Sf9 cells [MEKK3 (11–626)]. Control assays indicated that this preparation of purified MEKK3 could reconstitute JNK activation as determined by c-Jun phosphorylation only in the presence of MKK7, confirming its specificity *in vitro* (Figure 5A), and that free GST is not a substrate for MEKK3 (Figure 5B). *In vitro* kinase assays also revealed that GST-PA28 γ was directly phosphorylated by the purified preparation of MEKK3 (Figure 5B). A smaller protein in the GST-PA28 γ preparation, most likely a degradation product of the intact fusion protein, was resolved from the background phosphorylation and also phosphorylated by MEKK3 (Figure 5B). Western blotting revealed that both phosphoproteins were recognized by an antibody directed against PA28 γ protein (results not shown). In addition, phosphorylation of both PA28 γ proteins was dependent on the concentration of MEKK3 (11–626) in the *in vitro* reaction (Figure 5B). Thus MEKK3 (11–626) is able to directly activate MKK7 and to phosphorylate PA28 γ *in vitro*. To our knowledge, this is the first description of a kinase capable of phosphorylating a PA complex subunit.

MEKK3 and PA28 γ co-localize in mitotic Cos-7 cells

In order to determine the intracellular distribution of MEKK3 and PA28 γ , Cos-7 cells were transfected with mammalian expression constructs encoding a GFP-PA28 γ and Flag-MEKK3 and visualized by fluorescence microscopy. MEKK3 expression was exclusively cytoplasmic, whereas PA28 γ was located in the nucleus (Figure 6). However, in Cos-7 cells mitotically arrested with nocodazole, both PA28 γ and MEKK3 were found

Figure 4 PA28γ expression is increased by co-expression of MEKK3 in Cos-7 cells

Cos-7 cells were transfected with plasmids encoding HA-MEKK3, Flag-MEKK3, HA-PA28γ, HA-tagged kinase inactive MEKK3 (MEKK3 KD) or a non-tagged region of MEKK3 containing the first 178 amino acids [MEKK3 (1-178)], in various combinations as indicated. If not indicated differently, 2 μg of each DNA construct was used for transfection. The total amount of transfected DNA was made equivalent with pCMV5 empty vector. HA-tagged proteins were detected in cell lysates using an anti-HA antibody, and MEKK3 (1-178) was detected using B130, an antiserum raised to the N-terminal regulatory domain of MEKK3. Native PA28γ and γ-tubulin were visualized by specific antisera. **(A)** MEKK3 increases co-expressed PA28γ protein levels in a MEKK3 concentration-dependent manner. **(B)** The concentration of endogenous PA28γ protein is increased in Cos-7 cells transiently overexpressing HA-MEKK3 or Flag-MEKK3. **(C)** The N-terminal 178 amino acid region of MEKK3 is not capable of increasing the PA28γ protein levels in Cos-7 cells or of blocking PA28γ induction by full-length MEKK3. **(D)** Kinase-inactive MEKK3 (MEKK3 KD) does not increase PA28γ protein expression.

throughout the cytoplasm (Figure 6). Similar redistribution during mitosis has been reported for *Drosophila* PA28γ [31] and was also observed in mitotic HeLa cells (results not shown), suggesting that PA28γ and MEKK3 may interact following nuclear envelope breakdown at this distinct stage of the cell cycle.

DISCUSSION

MEKK3 is a MAPKKK that regulates the stress-activated protein kinases JNK and p38 by direct phosphorylation of their activators MKK7 and MKK6 respectively [1]. To date, however, the mechanisms by which stress signals lead to MEKK3 activation have not yet been defined, and additional functions of MEKK3 remain to be discovered. It has been reported previously [29]

that 14-3-3ζ and 14-3-3ε can bind to MEKK3 without affecting the ability of MEKK3 to activate JNK. 14-3-3 proteins act as chaperones to allow conformational stabilization of kinases and as scaffolds to bring proteins together in a complex, thereby regulating the activity of kinases [32]. In the present study, we isolated 14-3-3β, γ, θ and η in addition to 14-3-3ζ and ε as MEKK3-binding partners, with 14-3-3ε being the most abundant protein detected in the screen. This implies that 14-3-3ε may play a major role in MEKK3 regulation or subcellular localization. The physiological function of this interaction will need to be defined by future experiments.

Another clone detected in the two-hybrid screen was PA28γ, a nuclear protein with sequence homology to PA28α and β [16]. PA28α and β form heteroheptameric complexes and are involved

Figure 5 MEKK3 phosphorylates PA28 γ *in vitro*

Purified recombinant MEKK3 (11–626) was incubated in the presence of 5 μ Ci of [γ - 32 P]ATP with the indicated GST fusion proteins. Phosphorylated proteins were resolved by SDS/PAGE and visualized by autoradiography. **(A)** MEKK3 (11–626) directly activates MKK7. MEKK3 activity was assessed in a coupled assay in which GST–c-Jun phosphorylation by GST–JNK1 was determined, as indicated, in the absence and presence of GST–MKK7 [4]. **(B)** MEKK3 (11–626) phosphorylates PA28 γ in a concentration-dependent manner. Phosphorylation of intact GST–PA28 γ is indicated, together with a lower-molecular-mass protein that represents a degradation product of GST–PA28 γ (arrow). Free GST is not phosphorylated by MEKK3.

Figure 6 Localization of MEKK3 and PA28 γ in Cos-7 cells

Flag–MEKK3 and GFP-tagged PA28 γ were co-expressed in Cos-7 cells and their intracellular distribution visualized by fluorescence microscopy. **(A)** Localization of DNA (blue), PA28 γ (green) and MEKK3 (red) in a single interphase cell. PA28 γ is exclusively nuclear, whereas the MEKK3 is exclusively cytoplasmic, as confirmed by the non-overlap of the fluorescent images (Merge). **(B)** Nuclear membrane breakdown and chromatin condensation (blue) in a nocodazole-arrested mitotic cell. Both MEKK3 (red) and PA28 γ (green) are localized in the cytoplasm as seen in the individual and merged fluorescence images.

in peptide antigen generation for presentation by MHC class I receptors [10,11]. PA28 γ forms homoheptameric complexes and acts as a potent activator of the trypsin-like activity of the proteasome *in vitro* [16]. The exact physiological function of PA28 γ is unknown, although its nuclear localization suggests that it does not contribute to antigen generation as part of the immune response. One report [19] suggests that PA28 γ may be involved

in the regulation of cell proliferation and growth based on the observations that PA28 γ -knockout mice display a modest growth retardation and that depletion of PA28 γ in embryonic fibroblasts impedes G₁/S-phase progression.

We reported recently [22] a direct interaction of PA28 α with B-Raf, a functional homologue of MEKK3 for the MEK–ERK module. This interaction involved the activation loop of PA28 α

located between amino acids 120 and 174 [22], a region highly conserved among the three PA28 proteins [33]. The sequence similarity between the activation loop of PA28 α and γ may explain why B-Raf interacted with both subunits (Figure 1). However, MEKK3 did not bind to PA28 α , indicating selectivity of MEKK3 for PA28 γ . The shortest PA28 γ clone detected in the screen lacked the N-terminal 106 amino acids, but retained the activation loop. Since MEKK3 interaction was selective for PA28 γ , it is unlikely to involve the same region of PA28 γ as that responsible for B-Raf binding. The sequences flanking the activation loop are different between PA28 isoforms [33] and may be responsible for MEKK3 binding. Further analysis will establish the domain within PA28 γ that is required for MEKK3 interaction.

Both MEKK3 and B-Raf consist of an N-terminal regulatory domain and a C-terminal kinase domain [1,32], although they do not share significant overall sequence similarity. Direct two-hybrid tests showed that the interaction with PA28 γ occurs within the extreme N-terminal domains of B-Raf and MEKK3 [22] (Figure 1), suggesting that the interactions may have common regulatory features. Furthermore, the β -galactosidase filter assay suggested that the affinity of PA28 γ binding was greater for MEKK3 (1–366) than for full-length MEKK3 (Figure 1A). This suggests that the MEKK3 kinase domain interferes with PA28 γ binding. For Raf, a regulatory 'back-fold' model has been proposed [32] in which the N-terminal regulatory half of the protein interacts with its C-terminal kinase domain. This inactive conformation is likely to be stabilized by 14-3-3 proteins and prevent binding of other factors to domains within the closed complex. It is possible that activation of B-Raf and MEKK3 involves a common process in which opening of the protein kinase allows interaction with regulatory factors and substrates.

The interaction of PA28 γ and MEKK3 detected in direct two-hybrid tests was confirmed using other methods. Specifically, GST-pulldown assays showed that PA28 γ -binding occurs within the N-terminal regulatory domain of MEKK3 (Figure 2), and co-immunoprecipitation experiments suggested that the interaction of full-length MEKK3 and PA28 γ can occur in mammalian cells (Figure 3). Furthermore, expression of recombinant PA28 γ in Cos-7 cells was markedly increased by co-expression of catalytically active MEKK3, but not by kinase-inactive MEKK3 or by the N-terminal MEKK3 domain responsible for PA28 γ binding (Figure 4). Importantly, native PA28 γ expression was also elevated in Cos-7 cells overexpressing MEKK3 alone (Figure 4). Although MEKK3 may induce endogenous PA28 γ protein expression by regulation of its promoter, it is unlikely to affect transcription of recombinant PA28 γ from the powerful CMV promoter, particularly because MEKK3 overexpression does not similarly affect other proteins expressed from pCMV5 (results not shown). Therefore it seems likely that MEKK3 stabilizes the PA28 γ protein by protecting it from degradation. This may be an important mechanism whereby MEKK3 contributes to proteasomal regulation by PA28 γ .

Activation of the proteasome requires binding of regulatory protein complexes to both ends of the 20 S core complex. This interaction allows proteins to be recognized by the regulatory subunits and to enter the internal catalytic chamber to be degraded [34,35]. Since the 20 S proteasome is found well in excess of activator complexes and free activator complexes have not been detected, proteasomal activity is likely to be primarily regulated by changes in activator complex concentrations [9,36,37]. Indeed, IFN- γ strongly induces expression of PA28 α and β mRNA and protein levels, leading to enhanced antigen-presentation by cells [10,11]. However, IFN- γ has little effect on PA28 γ expression [7] and may even decrease it. For example, in human SW620

cells, IFN- γ does not appear to affect PA28 γ mRNA expression, but causes a complete disappearance of PA28 γ protein [18]. Similarly, infection of mouse liver with diverse bacteria or viruses causes disappearance of PA28 γ within 8 days of infection [38]. In the present study, MEKK3 overexpression in Cos-7 cells increased PA28 γ expression, and this effect was dependent on the kinase activity of MEKK3 (Figure 4). In cells, PA28 α , β and γ are present as phosphoproteins, as is the 20 S core complex [20,39,40]. The core-complex subunits C8 and C9 are phosphorylated most probably by protein kinase CK2 (formerly casein kinase II) [39–41], and dephosphorylation interferes with PA700 association, suggesting that phosphorylation has a regulatory function [40]. It has also been reported [20] that dephosphorylation of PA28 α and β abolishes their activity, although others have not seen such an effect, since bacterially purified PA28 remains fully active *in vitro* ([16], and results not shown). Whether phosphorylation of PA28 proteins is important for their activity *in vivo* remains to be established. However, MEKK3 recognizes PA28 γ as an *in vitro* substrate (Figure 5) and represents the first description of a protein kinase capable of phosphorylating a PA subunit. Furthermore, *in vitro* assays using purified components suggested that MEKK3 may enhance the trypsin-like activity of the PA28 γ -dependent 20 S proteasome (results not shown), although further studies will be required to establish whether phosphorylation of PA28 γ by MEKK3 may also contribute to this effect.

Since the catalytic activity of MEKK3 was necessary to increase the concentration of PA28 γ in cells, phosphorylation of PA28 γ by MEKK3 may serve to activate the proteasome by increasing PA28 γ stability and 20 S–PA28 γ complex formation, rather than by altering intrinsic PA28 γ activity. It is also possible that phosphorylated PA28 γ serves a function other than activation of the 20 S proteasome. For example, cytoplasmic PA28 α and β can act as cofactors for protein refolding in addition to their function as 20 S activators [42], and it seems likely that distinct functions of nuclear PA28 γ have yet to be discovered.

The present study demonstrates that MEKK3 and PA28 γ are capable of direct interaction and that the kinase activity of MEKK3 can increase PA28 γ protein expression in intact cells. However, MEKK3 is located in the cytoplasm, whereas PA28 γ is exclusively nuclear [43]. Clearly, in order to serve a common function, they have to interact with each other in the cell. One possibility is that MEKK3 phosphorylates newly synthesized PA28 γ in the cytosol, possibly to facilitate its nuclear transport or its assembly into heptameric complexes. Alternatively, interaction of PA28 γ and MEKK3 may be cell-cycle stage-dependent. To examine this possibility, the distribution of the proteins was assessed in an asynchronous population of cells and in cells mitotically arrested with nocodazole. Following nuclear envelope breakdown of mitotic cells, there was a redistribution of nuclear PA28 γ throughout the cell, thereby providing an opportunity for the protein to interact with MEKK3 in the cytoplasm (Figure 6). It is interesting to speculate from these results that MEKK3 and PA28 γ may only interact during mitosis, possibly to facilitate phosphorylation and stabilization of PA28 γ at this specific cell-cycle stage, where proteasome-dependent destruction of cell-cycle proteins is a key requirement for cells to exit mitosis [44,45]. Indeed, recent results from PA28 γ -knockout studies support a role for PA28 γ in cell-cycle control [19]. Although the physiological functions of PA28 γ remain to be established, our observations suggest a potential mechanism to couple a stress kinase signalling pathway with proteasomal regulation.

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