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ORIGINAL ARTICLE

Septin 8 is an interaction partner and *in vitro* substrate of MK5

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Abstract

AIM: To identify novel substrates for the mitogen-activated protein kinase-activated protein kinase 5 (MK5).

METHODS: Yeast two-hybrid screening with MK5 as bait was used to identify novel possible interaction partners. The binding of putative partner was further examined by glutathione S-transferase (GST) pull-down, co-immunoprecipitation and fluorescence resonance energy transfer (FRET) analysis. *In vitro* kinase and peptide array assays were used to map MK5 phospho-acceptor sites on the new partner. Confocal microscopy was performed to study the subcellular localization of MK5 and its partners.

RESULTS: Septin 8 was identified as a novel interaction partner for MK5 by yeast two-hybrid screening. This interaction was confirmed by GST pull-down, co-immunoprecipitation and FRET analysis. Septin 5, which can form a complex with septin 8, did not interact with MK5. Serine residues 242 and 271 on septin 8 were identified as *in vitro* MK5 phosphorylation sites. MK5 and septin 8 co-localized in the perinuclear area and in cell protrusions. Moreover, both proteins co-localized with vesicle marker synaptophysin.

CONCLUSION: Septin 8 is a bona fide interaction partner and *in vitro* substrate for MK5. This interaction may be implicated in vesicle trafficking.

Key words: Mitogen-activated protein kinase-activated protein kinase-5; Fluorescence resonance energy transfer; Septin; Phosphorylation; Synaptophysin

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INTRODUCTION

The group of mitogen-activated protein kinases (MAPKs) participates in control of several vital processes, including metabolism, transcription, apoptosis, cell cycle progression, differentiation, cytoskeletal rearrangement and cell movement. MAPK-activated protein kinase 5 (MK5), also designated PRAK, belongs to the group of kinases downstream of MAPK, originally identified as *in vitro* p38 MAPK substrate[1,2]. Biological functions of MK5 are poorly understood despite the high similarity in structure with other widely studied members of the subgroup[3]. Recent studies have highlighted the importance of MK5

in ras-induced senescence, antiproliferation, tumor suppression, anxiety-related behavior, energy-depletion-induced suppression of mammalian target of rapamycin C1, rearrangements of the cytoskeleton, endothelial cell migration, and tumor angiogenesis[4-9]. Several *bona fide* substrates have been identified, including extracellular signal-regulated kinase (ERK)3, ERK4, 14-3-3ε, p53, Ras homolog enriched in brain (Rheb) and heat shock protein (Hsp)27[6,8,10-15]. The role of MK5 in actin architecture involves its link with 14-3-3ε and Hsp27[14,15], while MK5-mediated phosphorylation of p53 at Ser-37 results in increased transcription of $p21^{Cip1}$, and inhibition of cell proliferation[6,16]. The biological relevance of MK5-ERK3 and MK5-ERK4 interactions remains unknown.

Septins belong to a group of small GTPases that were originally described in the budding yeast *Saccharomyces cerevisiae* as a group of cell division cycle regulatory genes. Proteins were isolated in yeast through the analysis of temperature-sensitive mutants showing impaired cytokinesis and budding[17]. Their role in formation of the septum between mother and daughter yeast cells is the background for the given name septins[17,18]. Septins are highly conserved and have been identified in most cell types of multicellular organisms[19]. Fourteen septin genes (*SEPT1-SEPT14*) have been characterized to date in humans[20]. Septin genes undergo alternative splicing and produce multiple protein products that are expressed at different ratios in different tissues[20]. These GTP-binding proteins are involved in diverse processes such as cytokinesis, membrane remodeling and compartmentalization, cytoskeleton rearrangement, vesicle trafficking, and apoptosis[18,20,21]. Knockout mice have been generated for *SEPT3, 4, 5, 6, 7* and *9* genes[22]. $SEPT7^{-/-}$ and $SEPT9^{-/-}$ mice were embryonic lethal, while $SEPT3^{-/-}$ and $SEPT6^{-/-}$ mice had no obvious phenotype[23,24]. $SEPT4^{-/-}$ mice had defects in behavior and reproduction, while $SEPT5^{-/-}$ animals had elevated platelet hypersensitivity[25-27]. Septins have been reported to be perturbed in various human diseases, including neurological disorders, infection, and neoplasia[28].

SEPT8 was first identified as an interaction partner for SEPT5 by yeast two-hybrid screening[29], and is an alternatively spliced septin with 14 known transcripts and 10 different isoforms that are expressed in a variety of human tissues[20]. SEPT-SEPT interaction studies using yeast two- and three-hybrid assays revealed heterodimeric SEPT2:SEPT8, SEPT3:SEPT8, SEPT4:SEPT8, SEPT7:SEPT8 and SEPT9:SEPT8 complexes and heterotrimeric SEPT2:SEPT8:SEPT7, SEPT4:SEPT8:SEPT7, SEPT5:SEPT8:SEPT7, SEPT2:SEPT8:SEPT9, SEPT4:SEPT8:SEPT9, SEPT5:SEPT8:SEPT9 complexes[30]. SEPT8 together with SEPT4 and SEPT5 have been designated platelet septins due to high expression and interaction in these cells. Moreover all three septins are highly expressed in brain and heart tissues as well as in prostate, testis and ovary[31]. In platelets, they have marked preference for areas surrounding α-granules. Activation of platelets leads to translocation of SEPT4 and SEPT8 to the platelet surface[32]. SEPT5 is involved in exocytosis (serotonin release) in platelets and may regulate synaptic vesicle dynamics[25,33-35]. SEPT8 is expressed in rat brain and expression is developmentally regulated[36]. Besides septin proteins, other proteins have been shown to interact with SEPT8. Vesicle-associated membrane protein (VAMP)2 and syntaxin1A have been identified as binding partners for SEPT8. Furthermore, SEPT8 in rat brain disrupts the binding of VAMP2 to synaptophysin. These results suggest a possible involvement of SEPT8 in the regulation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex formation and subsequent exocytosis[36], but experimental proof is lacking and the biological role of SEPT8 remains unknown.

To increase the understanding of biological functions of MK5, we used the yeast two-hybrid system and identified SEPT8 as a new interaction partner. SEPT8 appears to be phosphorylated by MK5 *in vitro*. These two proteins showed co-localization in the perinuclear area or protrusions of the cells as well as co-localization with synaptophysin in a neuroblastoma cell line. These results support previous assumptions on the role of SEPT8 in exocytosis and may indicate a possible involvement of MK5 in regulated secretion through phosphorylation of SEPT8.

MATERIALS AND METHODS

Yeast two-hybrid screening

Yeast two-hybrid screening of MK5 was performed in yeast PBN204 strain containing three reporters (*URA3*, *lacZ*, and *ADE2*) that are under the control of different *GAL* promoters. Yeast transformants of the MK5 bait and human brain cDNA AD library were spread on selection medium (SD-leucine, tryptophan, uracil; SD-LWU) that supports growth of yeast with bait and prey plasmids yielding proteins interacting with each other.

Antibodies

The anti-PRAK (A7) and anti-SEPT8 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Anti-synaptophysin fluorescein isothiocyanate (FITC) conjugate (SY38) antibodies were purchased from Progen Biotechnik GmbH (Heidelberg, Germany). The alkaline-phosphatase-conjugated secondary antibodies sheep anti-mouse IgG and anti-rabbit IgG were from Sigma Aldrich (St. Louis, MO, United States).

Plasmid construction and mutagenesis

The plasmid pRK5-flag-Sept8 was a kind gift from Dr. Koh-Ichi Nagata and Dr. Masaki Inagaki (Aichi Cancer Center Research Institute, Nagoya, Japan)[37]. pGEX-Sept8 and AsRed-Sept8 plasmids were generated by digesting pRK5-flag-Sept8 with *Eco*RI and *Xho*I or *Bsp*EI and *Xho*I, respectively, and ligation into corresponding vectors, namely pGEX-4T-1 (GE Healthcare, Oslo, Norway) and AsRed-C1 (BD Biosciences, Trondheim, Norway). All plasmids were verified by sequencing. The MK5 expression plasmid has been previously described[38]. The oligonucleotides 5'-GCAGTCGACATGGCGGCCACCGACCTGGA-3' and 5'-TATGCGGCCGCTTAATTCTTCTTGTCCTTG-3' were used to amplify

SEPT8-fragment from pRK5-Flag-Sept8. Then, the fragment of SEPT8 was cut with *Sal*I/*Not*I and ligated into the corresponding sites of pENTR1A (Invitrogen, Dynal AS, Oslo, Norway). The pEGFP-MK5 was digested with *Sma*I/*Eco*RI, and the fragment ligated into the corresponding sites of pBluescript SK plus. The resulting pBluescript-MK5 construct was then digested with *Eco*RI/*Not*I, and the fragment ligated into the corresponding sites of pENTR2B (Invitrogen). The pcDNA-CREB (cAMP response element-binding protein) plasmid was digested with *Kpn*I/*Not*I, and the fragment ligated into the corresponding sites of pENTR3C (Invitrogen). The resulting pENTR-SEPT8, pENTR-MK5 and pENTR-CREB clones were further recombined with Gateway destination vectors pDest-EYFP and pDest-ECFP (a gift from Dr. Terje Johansen, University of Tromso, Norway) generating EYFP-SEPT8, ECFP-SEPT8, EYFP-MK5, ECFP-MK5, EYFP-CREB and ECFP-CREB.

Glutathione S-transferase-fusion protein purification

Glutathione S-transferase (GST) fusion proteins were purified from *Escherichia coli* (*E. coli*) BL21 extracts using glutathione-agarose beads as previously described[38], and the GST moiety was removed from the GST beads by use of thrombin according to the instructions of the manufacturer.

In vitro kinase assays

Peptide arrays were synthesized on cellulose paper by using MultiPep automated multiple peptide synthesizer (INTAVIS Bioanalytical Instruments, Koln, Germany). Successive 20-mer peptides spanning the complete SEPT8 protein were synthesized. Each consecutive peptide had a three-amino-acid shift compared to the previous peptide. For phosphorylation, membranes were briefly wet in ethanol prior to incubation in phosphorylation buffer [20 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (DTT), and 0.2 mg/mL bovine serum albumin (BSA)] for 1 h at room temperature. Thereafter, the array was incubated at 4 ℃ overnight in blocking buffer (phosphorylation buffer containing 1 mg/mL BSA and 100 μmol/L ATP). Phosphorylation was initiated by incubating the membrane in the presence of activated MK5 (Millipore Upstate, Billerica, MA, United States) in 20 mL phosphorylation buffer containing 50 μmol/L ATP and 0.37 MBq of [γ-^{32}P] ATP for 30 min at 30 ℃ with agitation. The membrane was washed extensively in 1 mol/L NaCl followed by washes in 5% H_3PO_4 and dried down[39]. The phosphorylated membrane was then visualized by the FUJIFILM BioImaging Analyser (type BAS-5000). *In vitro* phosphorylation of SEPT8 by activated MK5 was performed in 25 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $MgCl_2$, 0.05 mg/mL BSA, 2.5 mmol/L DTT, 0.15 mmol/L cold ATP, and 0.3 μL [γ-^{32}P] ATP (3000 Ci/mmol; GE Healthcare) in a total volume of 40 μL at 30 ℃ for 1 h. The reaction was stopped in 4 × lithium dodecyl sulfate (LDS) Sample buffer, and proteins were denatured at 70 ℃ for 10 min. The phosphorylation was analyzed on NuPAGE 4%-12% BisTris SDS-PAGE (Invitrogen) for 50 min at 200 V and then subjected to autoradiography.

Cell culture and transfections

The human neuroblastoma cell line SK-N-DZ (CRL-2194) was from ATCC (LGC Promochem, Middlesex, United Kingdom), while PC12 cells were purchased from the European Collection of Cell Culture (ECACC; Wiltshire, United Kingdom). The cells were maintained in RPMI 1640, supplemented with 10% horse serum (Gibco) and 5% fetal bovine serum, 2 mmol/L L-glutamine, penicillin (110 U/mL) and streptomycin (100 μg/mL). Cells were transfected using the Nucleofection kit (Amaxa) according to the manufacturer's instructions. HEK293 cells (ATCC CRL-1573) and HeLa cells (ATCC CCL-2) were purchased from the American Type Culture Collection (LGC Standards, Boras, Sweden) and maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA, United States), 2 mmol/L L-glutamine, (110 U/mL) and streptomycin (100 μg/mL). HeLa cells also received 1 × nonessential amino acids (Invitrogen). HeLa cells were transfected with Lipofectamine 2000 (Invitrogen).

Co-immunoprecipitation

HEK293 cell extracts were harvested and lysed in buffer containing 20 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 5 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 0.27 mol/L sucrose, 10 mmol/L β-glycerophosphate, and complete protease inhibitor cocktail (Roche Applied Sciences, Oslo, Norway). Lysates were cleared by centrifugation at 4 ℃ for 10 min at 15 000 *g*. Lysates were incubated with the appropriate antibody for at least 1 h at 4 ℃, before addition of 60 μL slurry [i.e., 30 μL protein G-agarose (GE Healthcare) equilibrated with 30 μL lysis buffer] and incubated for an additional 1 h. The immunoprecipitates were then washed three times in lysis buffer and twice in 50 mmol/L Tris-HCl, pH 8.0. Twenty microliters of 2 × LDS sample buffer were added to the beads before denaturation at 70 ℃ for 10 min. The immunoprecipitates were analyzed on western blotting.

Fluorescent microscopy and fluorescence resonance energy transfer

Cells with ectopically expressed proteins fused with green fluorescent protein or red fluorescent proteins were analyzed directly using confocal laser scanning microscope (LSM 510 META; Zeiss, Oslo, Norway). For fluorescence resonance energy transfer (FRET) analysis, HeLa cells were cotransfected with ECFP-MK5 and EYFP-SEPT8 or EYFP-CREB using TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, United States). Then, 24 h after transfection, the cells were fixed with 4% formaldehyde. FRET analysis was carried out with a Leica TCS SP5 confocal microscope with a × 63, 1.2 W objective. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were excited with separate laser channels of 458 and 514 nm, respectively. Emission fluorescence intensity data was obtained at 465-500 nm (CFP) and 525-600 nm (YFP). CFP and YFP emission

signals were captured before and after 50% photobleaching YFP. FRET is indicated as the relative increase in CFP emission following YFP photobleaching. The imaging system was controlled by the Leica Application Suite Advanced Fluorescence software (http://www.leicamicrosystems.com).

Western blotting

For detection of coimmunoprecipitates and SEPT8, samples were analyzed by SDS-PAGE on NuPAGE 4%-12% BisTris SDS-PAGE gels from Invitrogen according to the manufacturer's protocol and blotted onto a 0.45-μm polyvinylidene difluoride membrane (Millipore). Immunoblotting was performed by first blocking the membrane with phosphate buffered solution (PBS)-T [PBS with 0.1% Tween 20 (Sigma) containing 10% (w/v) dried skimmed milk for 1 h and probed either by anti-PRAK or anti-SEPT8]. After three washes, the membrane was incubated with the appropriate secondary antibody for 1 h. Visualization of proteins was achieved by using CDP Star substrate (Tropix, Bedford, MA, United States) and Lumi-Imager F1 from Roche Applied Sciences. MagicMark™ western standard was from Invitrogen Life Technologies.

GST pull down

Cell lysate was precleared with 25 mg GST and 50 mL 50% slurry of glutathione-agarose beads for 2 h at 4 ℃. An input control was taken, and the cell lysate was divided into two tubes and 10 mg GST or GST-SEPT8 were added. The GST proteins were recovered after addition of glutathione beads and washed extensively in PBS-T. The proteins were eluted from the beads by addition of 20 mL loading buffer (LDS-buffer with 100 mmol/L DTT) followed by 10 min incubation at 70 ℃. The GST proteins were subsequently subjected to immunoblot analysis.

Densitometry

Densitometry was performed using a BioRad Model GS-700 Imaging Densitometer (Oslo, Norway) and the Multi-Analyst version 1.1 Software.

RESULTS

Screening of proteins that interact with MK5

To elaborate the biological functions of MK5, we decided to identify novel substrates for MK5. A yeast two-hybrid screen with MK5 as bait and a brain cDNA library was designed. After selecting yeast colonies on uracil-deficient medium, the activity of β-galactosidase was monitored. The URA^+ and $lacZ^+$ colonies were also tested for whether they could grow on adenosine-deficient medium. To maximize the specificity of the screening, protein-protein interaction was tested using three independent reporters with different types of GAL4-binding sites. This procedure eliminates interactions that result from nonspecific promoter activation. In order to confirm the interaction, the prey plasmids from 29 candidates that expressed at least two reporters were selected by *E. coli* transformation, and then the candidate prey plasmids were reintroduced into yeast with MK5 bait plasmid or with a negative control plasmid expressing GAL4-binding domain but lacking the bait part. Twenty real positives were identified that encoded three different proteins. Among 20 real positives, 17 preys contained the same cDNA encoding SEPT8_v2 (AF440762). Two other positive clones encoded the previously reported MK5 interaction partner ERK3[10,11].

SEPT8 binds to MK5 in vitro and in vivo

To verify the interaction observed in the yeast two-hybrid system, GST pull-down was performed. Lysates of HEK293 cells transfected with the expression plasmid for GFP-MK5 were incubated with glutathione-sepharose beads accompanied by either GST alone or GST-SEPT8. After immunoprecipitation, MK5 was detected by western blotting using anti-PRAK antibody. The results showed that MK5 could bind GST-SEPT8 (Figure 1A) but not GST alone.

To establish whether complexes of MK5 and SEPT8 existed within mammalian cells, HEK293 cells were co-transfected with MK5 and SEPT8 expression plasmids because these cells give high transfection efficiency. Reciprocal coimmunoprecipitation studies were conducted with anti-PRAK antibody followed by western blotting with anti-SEPT8 antibody, or immunoprecipitation with anti-SEPT8 antibody followed by western blotting with anti-PRAK antibody. Specific interaction was monitored between the two proteins (Figure 1B and C). The signal in the last lane in Figure 1C (lysate of cells expressing ectopically expressed SEPT8 but not MK5) was due to interaction of SEPT8 with endogenous MK5. No interactions were visualized in the control precipitation using vehicles. To detect interaction between endogenous MK5 and SEPT8, PC12 cells were used because both MK5 and SEPT8 are relatively highly expressed in neural cells[1,2,36]. MK5 could be detected when endogenous SEPT8 was immunoprecipitated (Figure 1D). Combined, our results clearly demonstrate that MK5 and SEPT8 specifically interact *in vitro* and *in vivo*. SEPT8 was originally identified as an interaction partner for SEPT5[29], therefore, we tested whether MK5 and SEPT5 complexes were found in cells. Coimmunprecipitation studies failed to detect MK5:SEPT5 complexes (results not shown).

We performed FRET analysis to monitor interaction between MK5 and SEPT8 in cells. HeLa cells were transfected with expression plasmids for MK5 and SEPT8. As a negative control, we included CREB because it does not interact with MK5[4]. After photobleaching, interaction between MK5 and SEPT, but not MK5 and CREB, was observed as indicated by a relative increase in CFP emission following YFP photobleaching (Figure 2).

MK5 phosphorylates SEPT8 in vitro

In previous studies, several septins have been shown to be phosphorylated *in vitro* and/or *in vivo,* including SEPT1, SEPT2, SEPT3 and SEPT5[40-44]. After describing the interaction between two proteins, we wanted to know whether MK5 possesses catalytic activity towards

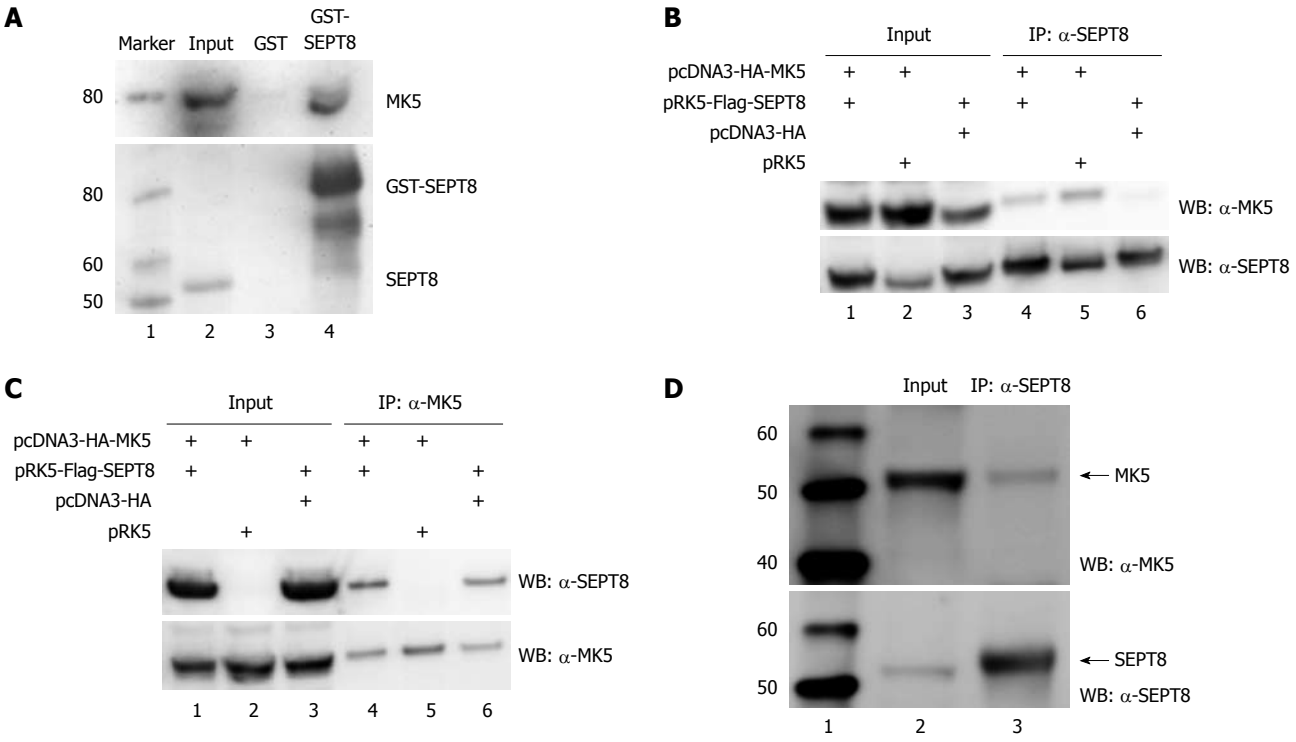

Figure 1 Mitogen-activated protein kinase-activated protein kinase 5 and SEPT8 interact *in vitro* and *in vivo*. A: Glutathione S-transferase (GST) pull-down assay. GST-SEPT8 or GST alone purified from *Escherichia coli* and immobilized on glutathione-Sepharose beads were incubated for 60 min with lysate of HEK293 cells transfected with GFP-mitogen-activated protein kinase-activated protein kinase 5 (MK5). After washing the beads five times, bound proteins were eluted by boiling, subjected to SDS-PAGE, and immunoblotted with anti-PRAK (upper panel) or anti-SEPT8 (lower panel) antibody. Lane 1: Protein molecular mass marker (in kDa); lane 2: Cell lysate; lane 3: Cell lysate after pull down with GST; lane 4: Cell lysate after pull down with GST-SEPT8; B: Coimmunoprecipitation of FLAG-SEPT8 and hemagglutinin (HA)-MK5. HA-MK5- and/or FLAG-SEPT8-encoding plasmids were transiently expressed in HEK293 cells. Total cellular lysates (input; lanes 1-3) and SEPT8 immunoprecipitates (IP: α-SEPT8; lanes 4-6) were probed with anti-PRAK (upper panel) and anti-SEPT8 (lower panel) antibody. Lane 1: Cell lysate of cells cotransfected with expression plasmids for HA-tagged MK5 and FLAG-tagged SEPT8; lane 2: Cell lysate of cells cotransfected with expression plasmids for HA-tagged MK5 and empty vector for SEPT8; lane 3: Cell lysate of cells cotransfected with expression plasmids for FLAG-tagged SEPT8 and empty vector for MK5; lane 4: Immunoprecipitated lysate of cells cotransfected with expression plasmids for HA-tagged MK5 and FLAG-tagged SEPT8; lane 5: Immunoprecipitated lysate of cells cotransfected with expression plasmids for HA-tagged MK5 and empty vector for SEPT8; lane 6: Immunoprecipitated lysate of cells cotransfected with expression plasmids for FLAG-tagged SEPT8 and empty vector for MK5; C: Coimmunoprecipitation of HA-MK5 and FLAG-SEPT8. HEK293 cells were transiently transfected with expression plasmids for HA-MK5 and/or FLAG-SEPT8. Total cellular lysates (input) and HA-MK5 immunoprecipitates (IP: α-MK5) were probed with anti-SEPT8 (upper panel) and anti-PRAK (lower panel) antibody. Lanes 1-3: Lysates from transfected cells; lanes 4-6: Immunoprecipitation of cell lysates. See (B) for details; D: Coimmunoprecipitation of endogenous MK5 and SEPT8. Endogenous SEPT8 was immunoprecipitated from PC12 cells with anti-SEPT8 antibodies and the precipitate was analyzed for the presence of MK5 by anti-PRAK antibodies. Lane 1: Protein molecular mass marker (in kDa); lane 2: Lysate of PC12 cells (= input); lane 3: Immunoprecipitation with SEPT8 antibodies. The bottom panel shows control western blot with SEPT8 antibodies. IP: Immunoprecipitation; WB: Western blotting.

SEPT8. To test this, GST-SEPT8 fusion protein was purified from bacteria and the GST moiety was enzymatically cleaved. SEPT8 was then incubated with commercially available active MK5 in an *in vitro* kinase assay. Phosphoproteins were fractionated by SDS-PAGE on a 4%-20% acrylamide gradient gel and visualized by autoradiography. As shown in Figure 3A, SEPT8 was phosphorylated by MK5. As previously reported, MK5 autophosphorylation activity of MK5 was observed[38]. An additional band, corresponding to phosphorylated SEPT8 was detected. These results demonstrate that MK5 can phosphorylate of SEPT8 *in vitro*.

Identification of phosphorylation sites on SEPT8

To identify MK5 phosphoacceptor sites on SEPT8, we performed an *in vitro* kinase assay using peptide arrays. One separate peptide, YFIT^{237}PT^{239}GHS^{242}LKS^{245}LDLVT^{250}MK K and two consecutive peptides IPIIAKADT^{269}IS^{271}KS^{273} ELHKFKI and IAKADT^{269}IS^{271}KS^{273}ELHKFKIKIM appeared phosphorylated (Figure 3B). Putative phosphoacceptor sites for the serine/threonine MK5 kinase are marked. We analyzed the SEPT8 sequence with NetPhos 2.0 (accessible on the internet: http://www.cbs.dtu.dk/services/NetPhos/). This software algorithm predicts the potential phosphorylation sites in proteins. According to the NetPhos description, scores close to 1 are the most likely to represent an actual phosphorylation site. Among all predicted sites, two serine residues in the sequences of peptides that were phosphorylated on peptide array had a high score, namely 0.994 for Ser-242 and 0.944 for Ser-271. We generated plasmids encoding GST-SEPTS242A, GST-SEPTS271A and GST-SEPTS242A/S271A in which these Ser residues were replaced by alanine and expressed these fusion proteins in bacteria. GST was enzymatically removed from the purified proteins and *in vitro* kinase assay with active MK5 was performed. Mutation of Ser-242 or Ser-271 into Ala reduced MK5-mediated phosphorylation of SEPT8, but the former substitution had less effect than

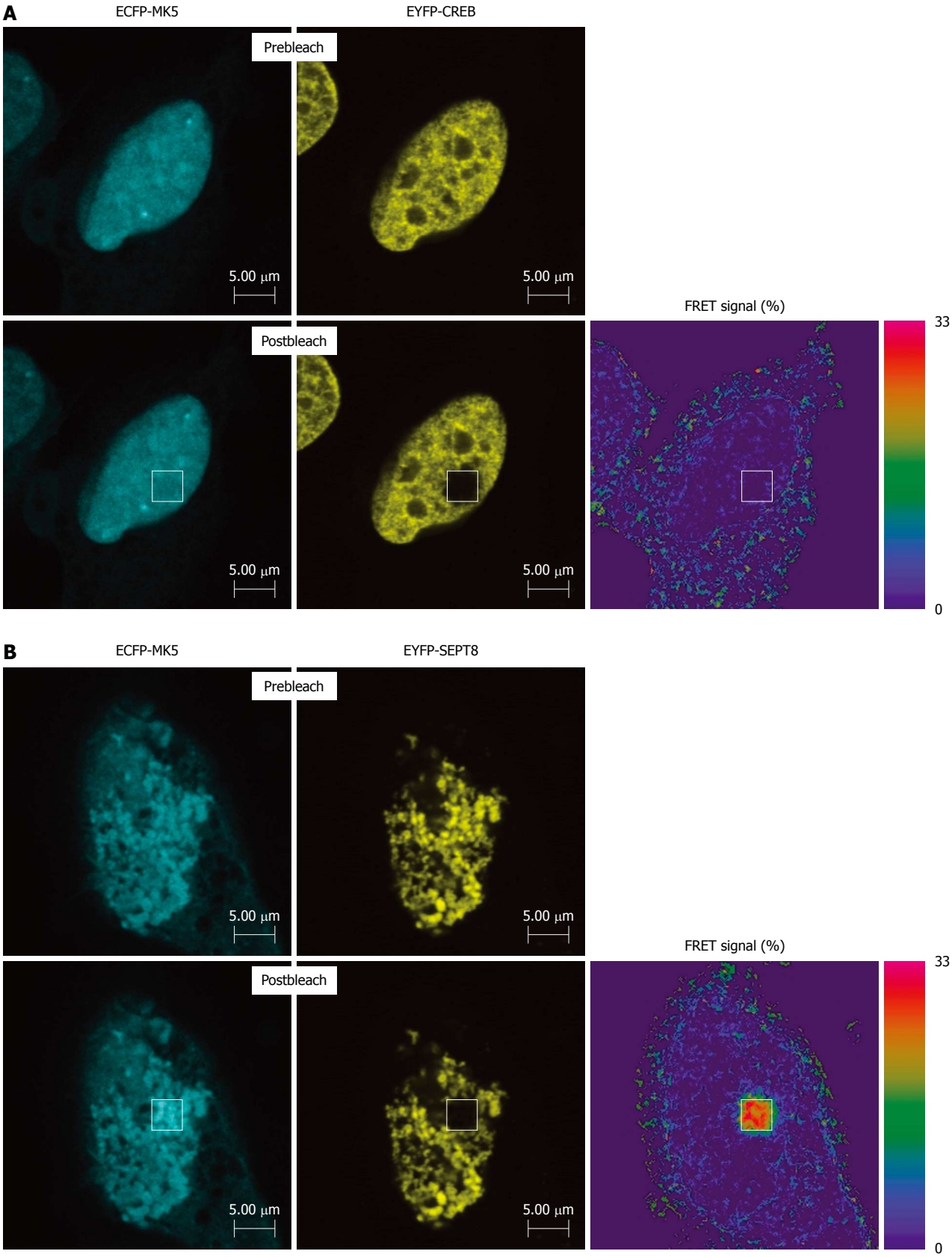

Figure 2 Fluorescence resonance energy transfer analysis shows interaction between mitogen-activated protein kinase-activated protein kinase 5 and SEPT8. (A) HeLa cells were cotransfected with expression plasmids for heat shock protein (CFP)-tagged mitogen-activated protein kinase-activated protein kinase 5 (MK5) and yellow fluorescent protein (YFP)-tagged CREB or (B) with expression vectors for CFP-tagged MK5 and YFP-tagged SEPT8. Twenty-four hours after transfection, cells were fixed and fluorescence resonance energy transfer (FRET) analysis was carried out. CFP and YFP were excited with separate laser channels of 458 and 514 nm, respectively. Emission fluorescence intensity data were obtained at 465-500 nm (CFP) and 525-600 nm (YFP). CFP and YFP emission signals were captured before and after 50% photobleaching YFP. FRET is indicated as the relative increase in CFP emission following YFP photobleaching.

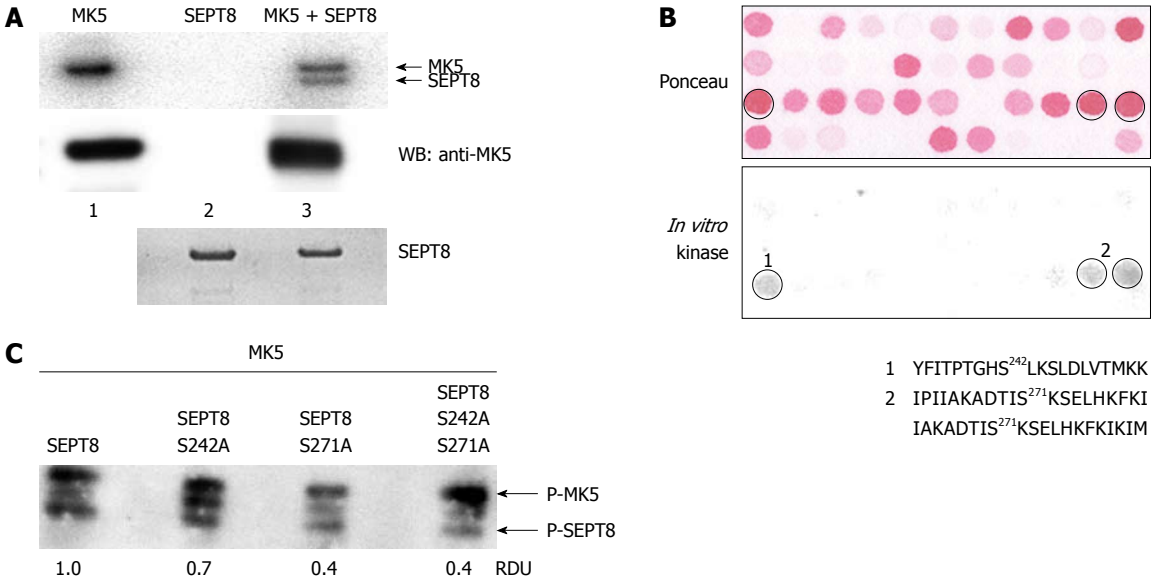

Figure 3 Mitogen-activated protein kinase-activated protein kinase 5 phosphorylates septin 8 *in vitro*. A: *In vitro* kinase assay on recombinant SEPT8. Glutathione S-transferase (GST)-SEPT8 fusion protein was purified from *Escherichia coli* and the GST moiety was removed by thrombin. SEPT8 was incubated with activated mitogen-activated protein kinase-activated protein kinase 5 (MK5) for 30 min at 30 ℃ in the presence of [γ-^{32}P] ATP. Proteins were separated by SDS-PAGE and phosphorylation was visualized by autoradiography (upper panel). Loading control for MK5 (middle panel) and SEPT8 (lower panel) was performed by western blotting with anti-PRAK and anti-SEPT8 antibodies, respectively. Lane 1: Recombinant activated MK5; lane 2: Purified SEPT8 protein; lane 3: recombinant MK5 and purified SEPT8; B: SEPT8 peptide array was subjected to *in vitro* phosphorylation by activated MK5. Each spot represents a 20-mer peptide fragment of SEPT8. The peptide fragments constitute the complete SEPT8 protein. The sequential peptide has a 17-amino acid overlap with the previous peptide. Several spots with peptides were detected by autoradiography (lower panel). Ser-242 and Ser-271 with highest prediction score 0.994 and 0.944, respectively by Netphos software, were chosen as possible phosphorylation sites in the peptide sequences. The upper panel represents Ponceau staining of the peptide array membrane used. The sequences of the peptides representing the positive spots are shown; C: *In vitro* phosphorylation of wild-type SEPT8, and SEPT8 mutants carrying a single amino acid substitution (Ser-242 into Ala or Ser-271 into Ala, respectively) or the double amino acid substitution Ser-242 and Ser-271 into Ala. The upper band represents autophosphorylated MK5, while the lower band is phosphorylated SEPT8. Relative densitometry units (RDU) of the bands representing phosphorylated SEPT8 are shown. The value obtain for wild-type SEPT8 was arbitrary set as 1.0 and the other values were related to this.

the latter on phosphoSEPT8 levels. The double SEPT8 S242A/S271A substitution did not further reduce the phosphorylation levels of SEPT8 (Figure 3C). These findings indicate that Ser-271 and to a lesser extend Ser-242 represent *in vitro* targets for MK5, but that additional amino acid residues can be phosphorylated by MK5 *in vitro*.

MK5 colocalizes with SEPT8 in HEK293 and PC12 cells

To examine cellular localization of MK5 with SEPT8, confocal microscopy was performed on HEK293 cells expressing GFP-MK5 and AsRed-SEPT8 fusion proteins separately (Figure 4A) or simultaneously (Figure 4B). As previously reported, GFP-MK5 protein resides predominantly in the nucleus, but can also be detected in the cytoplasm[38], while The RFP-SEPT8 protein is exclusively located in the cytoplasm (Figure 4A). Cotransfection with expression plasmid encoding tagged MK5 and SEPT8 demonstrated colocalization of these two proteins in the perinuclear area (Figure 4B). Several studies have shown the involvement of septins in neuronal transmission and exocytosis. Therefore, we wanted to test if a similar colocalization pattern of proteins could be seen in neuroendocrine PC12 cells. Colocalization was observed not only in cell bodies but also in neurite terminals of PC12 cells (Figure 4C). Interestingly, a previous study showed that SEPT5 was concentrated near the plasma membrane and in growth cones of PC12 cells[45].

MK5 and SEPT8 colocalize with synaptophysin in the neuroblastoma cell line SK-N-DZ

In a previous study, SEPT8 colocalized with the synaptic marker synaptophysin[36]. To examine the cellular localization of SEPT8 with synaptophysin in the neuroblastoma cell line SK-N-DZ, cells were transfected with AsRed-SEPT8. Protein was visualized directly, and anti-synaptophysin FITC conjugate antibodies were used to monitor synaptophysin localization. SEPT8 and synaptophysin showed strong colocalization (Figure 5A), confirming the findings by Ito and colleagues[36]. We then investigated whether MK5 could colocalize with synaptophysin because we had observed colocalization with SEPT8 in several cell lines. Indeed, MK5 colocalized with synaptophysin in a similar fashion as SEPT8 (Figure 5B).

DISCUSSION

Several genuine substrates have been identified for MK5 including ERK3, ERK4, 14-3-3ε, p53, Rheb and Hsp27[6,8,10-15]. However, the functions of MK5 remain elusive, and MK5-deficient mice appear normal or die at embryonic stage E11.5, depending on the genetic background of the mice[6,10,46]. In our search for other possible MK5 interaction partners that may provide a clue to the biological functions of MK5, we have identified SEPT8 as an *in vitro* MK5 substrate. The interaction was originally revealed by

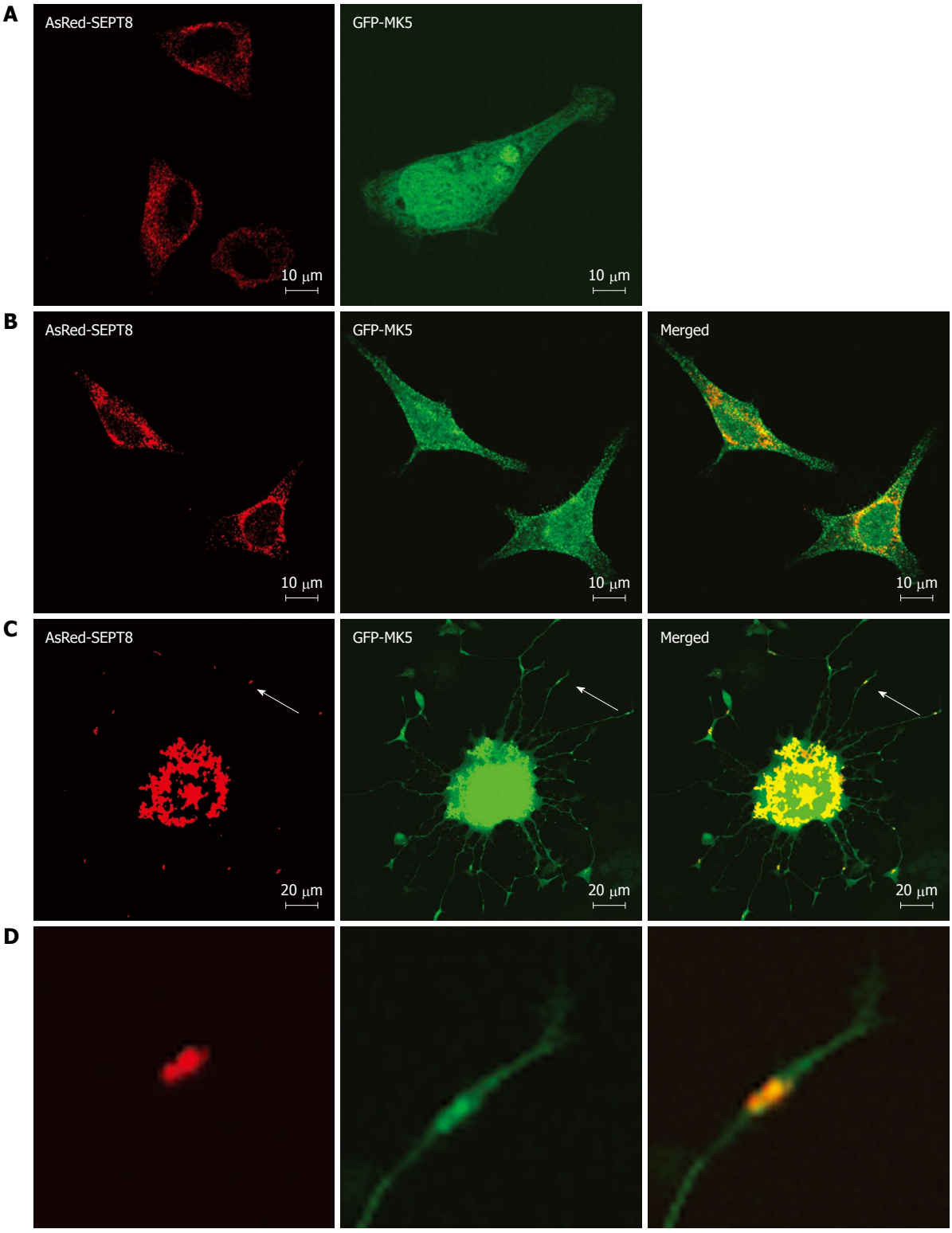

Figure 4 Mitogen-activated protein kinase-activated protein kinase 5 colocalizes with SEPT8. A: HeLa cells were transfected separately with an expression plasmid for GFP-mitogen-activated protein kinase-activated protein kinase 5 (MK5) (left panel) or AsRed-SEPT8 (right panel); B: HeLa cells cotransfected with both expression plasmids. The subcellular localization of RFP-tagged SEPT8 (left panel), GFP-tagged MK5 (middle panel), or both proteins (merged; right panel) is shown; C: PC12 cells cotransfected with expression vectors for AsRed-SEPT8 (left panel) and GFP-MK5 (middle panel). Colocalization is shown in the right panel; D: Enlargement of the areas marked by arrows in figures (C) shows clear colocalization of MK5 and SEPT8. After 24 h, cells were fixed and MK5 (green channel) and SEPT8 (red channel) were visualized directly.

yeast-two hybrid assay and confirmed in mammalian cell cultures by coimmunoprecipitation, GST pull-down and FRET assays. Moreover, colocalization of both proteins was observed in different cell lines transfected with expression plasmids for GFP-MK5 and AsRed-SEPT8 fusion. We went on to investigate whether MK5 had kinase

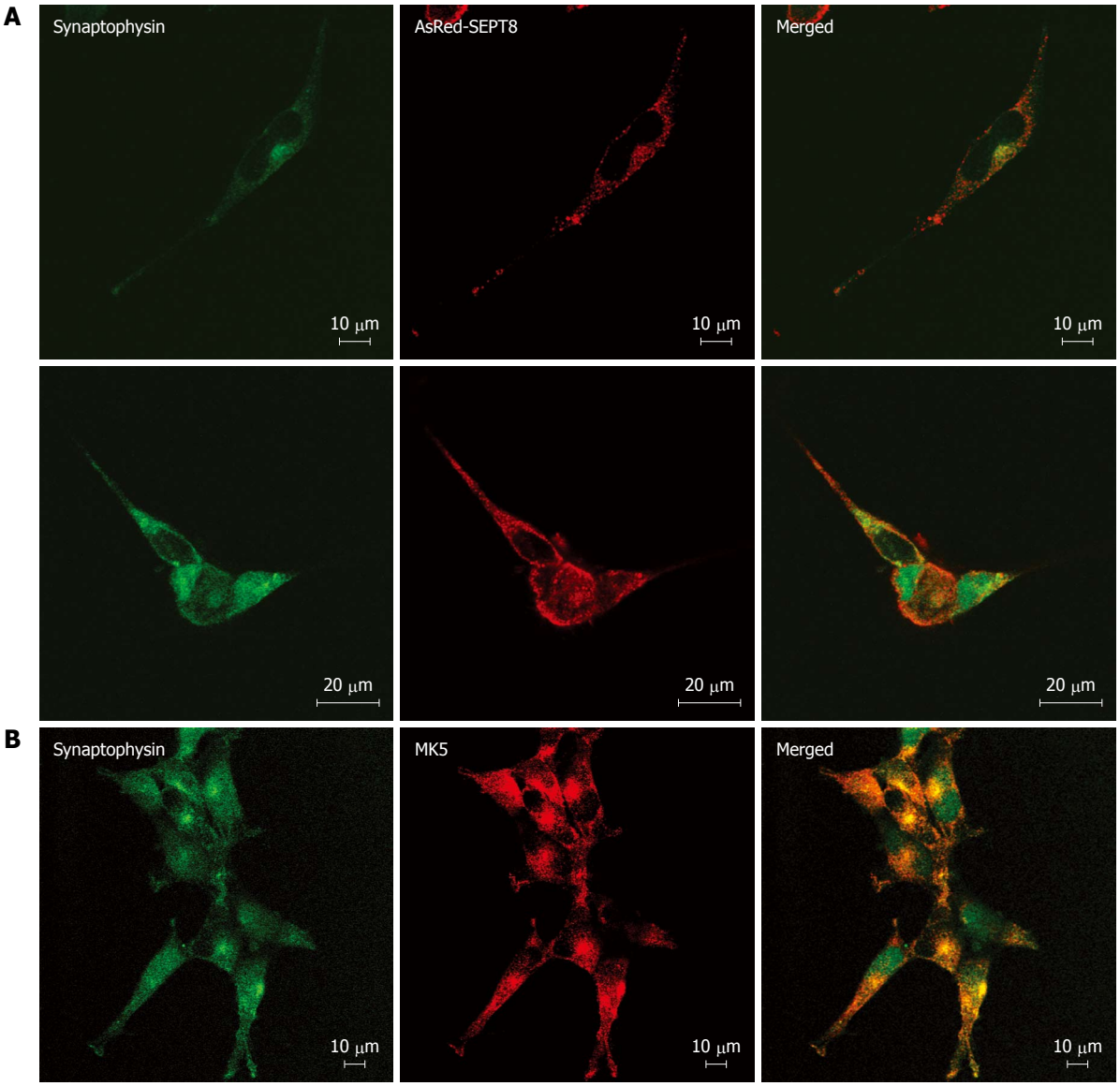

Figure 5 Mitogen-activated protein kinase-activated protein kinase 5 and SEPT8 colocalize with synaptophysin. A: SK-N-DZ cells were transfected with expression vector encoding AsRed-SEPT8 and after 24 h, cells were fixed. Synaptophysin was visualized by staining with anti-synaptophysin fluorescein isothiocyanate (FITC) conjugate antibody (left panels), while AsRed-SEPT8 was visualized directly (red channel; middle panels). A merged image of red and green channels is shown in the right panel; B: SK-N-DZ cells were fixed and stained with anti-synaptophysin FITC conjugate antibody (left panel) and with anti-PRAK antibody followed by Alexa Fluor 568 anti-rabbit antibody (middle panel). Merged image of red and green channels is shown in the right panel.

activity towards SEPT8 and it appeared that SEPT8 was indeed phosphorylated by MK5 *in vitro*. To the best of our knowledge, this is the first report linking MAPK-activated protein kinases to the group of septin proteins. However, previous studies have identified several mammalian septins as substrates of several kinases. For instance, SEPT1 has been shown to be phosphorylated *in vitro* by aurora-B kinase[41]. SEPT2 is a substrate of casein kinase 2, and its phosphorylation on Ser-218 is crucial for the proliferation of hepatoma carcinoma cells[44]. Moreover, SEPT2 is also an *in vitro* substrate for protein kinase (PK)C and cAMP-dependent PKA[47]. SEPT3 phosphorylation on Ser-91 by cGMP-dependent PKG may contribute to the regulation of its subcellular localization in neurons[40]. SEPT5 phosphorylation by cyclin-dependent kinase 5 at Ser-17 and Ser-327 reduces binding of septin to syntaxin, therefore playing a role in modulating exocytic secretion[42,43]. MK5 phosphorylates SEPT8 on Ser-271 and with lower stoichiometry on Ser-242 in an *in vitro* assay. The double SEPT8 S242A/S271A mutant is still phosphorylated *in vitro*, indicating that other sites may act as MK5 phosphoacceptor sites *in vitro*. Both sites are conserved in SEPT8 from different species ranging from fish to humans (Figure 6). This indicates that the sites are crucial for normal function of SEPT8. It remains to be tested whether MK5 can phosphorylate these sites *in vivo*. Phosphoproteomic studies have demonstrated that SEPT8 is phosphorylated on Ser-10 and Ser-141 in mouse brain, mouse skin melanoma, and nocodazole-arrested HeLa cells[48-50]. Both sites are conserved in fish, amphibians, and several mammals such as apes, primates, rodents and cattle. The protein kinases responsible for these phosphorylations have not

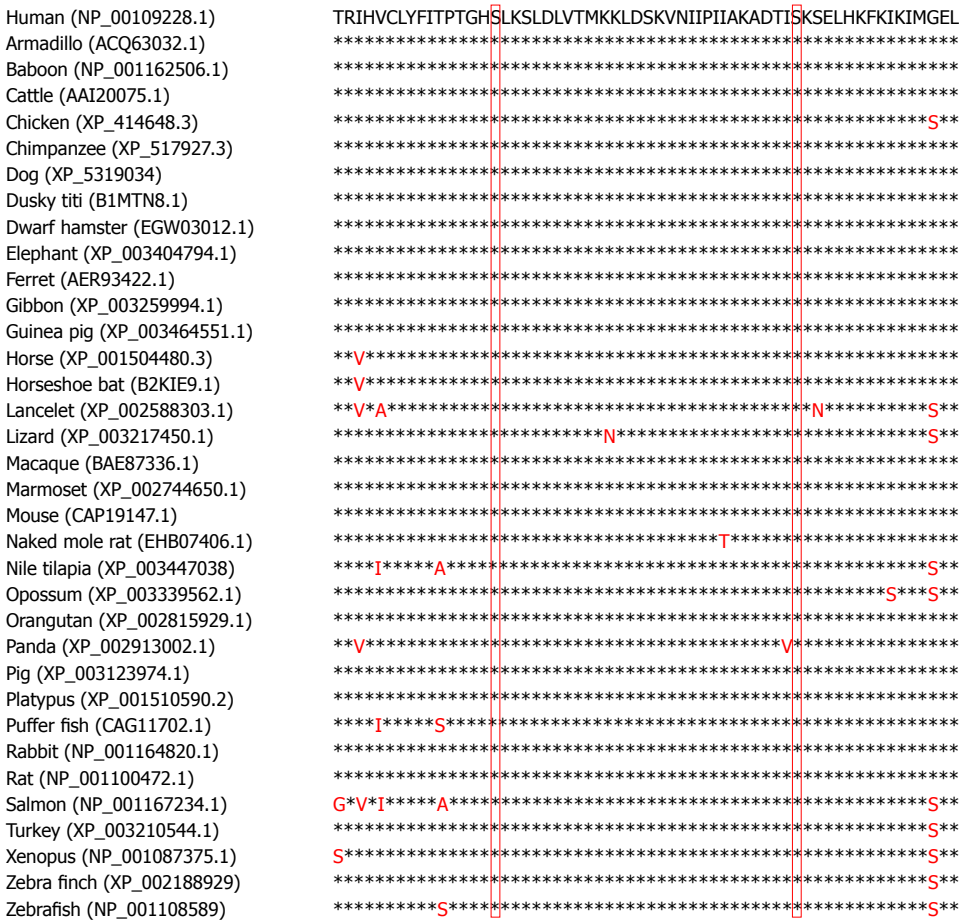

Figure 6 Mitogen-activated protein kinase-activated protein kinase 5 phosphoacceptor sites in human SEPT8 are conserved in SEPT8 from other species. Alignment of proven and putative SEPT8 proteins in different species of the region encompassing mitogen-activated protein kinase-activated protein kinase 5 phosphoacceptor sites Ser-242 and Ser-271 (numbering for human SEPT8). The code in parenthesis refers to the accession number in the Protein database (http://www.ncbi.nlm.nih.gov/protein/). Asterisk indicates identical amino acid residues. The non-conserved residues are shown in red. The one-letter amino acid code is used. The residues corresponding to human SEPT8 Ser-242 and Ser-271 are shown.

been identified. Our *in vitro* kinase assay with the peptide array did not indicate that Ser-10 and Ser-141 are *in vitro* phosphorylation sites for MK5.

The biological implications of MK5-mediated phosphorylation of SEPT8 are not known but it may affect structural properties by interfering with di- and trimeric complex formation with other septins. It has been shown for SEPT7 that substitution of tyrosine-318 into non-phosphorylatable Ala prevents interaction with SEPT8[30]. Phosphorylation of SEPT8 may have a similar effect on complex formation with septins. Phosphorylation may also affect the role of SEPT8 in cellular functions. The exact functions of SEPT8 remain to be elucidated. In accordance with SEPT5, SEPT8 is implicated in vesicular transport and exocytosis processes of neurotransmitter release and platelet secretion. In neurons, SEPT5 binds syntaxin, one of the SNARE proteins, and copurifies with synaptic vesicles, suggesting a role in exocytosis and synaptic vesicle dynamics[33,45,51]. Platelets from *SEPT5*$^{-/-}$ mice possess altered serotonin secretion and aggregation[25]. SEPT8 expression is high in neurons and platelets, and a recent study has suggested the importance of SEPT8 in the process of SNARE complex formation and subsequent neurotransmitter release, but actual experimental proof is lacking[32,34,36,45,52]. Moreover, SEPT8 colocalizes with the synaptic marker synaptophysin in primary cultured rat hippocampal neurons[36]. We also observed colocalization of MK5 and synaptophysin in neuroblastoma SK-N-DZ cells. MAPK p38, which can act as an upstream activator of MK5[53], has been shown to be involved in SNARE-dependent exocytotic release of brain-derived neurotrophic factor from microglia[54]. Finally, transgenic mice overexpressing an active variant of MK5 display different anxiety-related traits as compared to wild-type littermates[5]. All these observations point to a possible role of MK5 in exocytosis that may comprise SEPT8.

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COMMENTS

Background

The mitogen-activated protein kinase (MAPK) pathways transmit signals through a cascade of phosphorylation events, thereby controlling cellular processes such as proliferation, differentiation, migration, survival/apoptosis, and gene regulation. The precise function of one of the MAPKs, MAPK-activated protein kinase 5 (MK5), remains elusive. The aim of this study was to identify new substrates for MK5 in an effort to better understand the function of this protein kinase.

Research frontiers

Phosphorylation of proteins at serine, threonine and tyrosine residues by protein kinases is an important post-translational modification that controls the activity of the substrates. The human genome encodes more than 500 different protein kinases, including a large group known as MAPKs. MAPKs control through phosphorylation of their substrates crucial cellular processes. Perturbed action of these kinases can lead to malignant and nonmalignant diseases. It is therefore important to divulge the exact functions and genuine substrates of these kinases. This may allow designing therapeutic strategies for pathological conditions in which MAPKs play a causal role.

Innovations and breakthroughs

The genuine function of MK5 remains incompletely understood because few genuine substrates have been characterized. This study identified septin 8 as a novel interaction partner for MK5 and is the first to demonstrate that MAPKs can phosphorylate a member of the septin family. Septins are small GTP-binding proteins that participate in processes ranging from cytoskeletal architecture, scaffolding, cytokinesis, ciliogenesis, and neurogenesis. The function of septin 8 remains enigmatic, but it is enriched at presynapses and interacts with several proteins of synaptic vesicles. Hence, septin 8 may be involved in neurotransmitter release and the role of septin 8 may be modulated by MK5-mediated phosphorylation. Interestingly, transgenic mice overexpressing a constitutive active variant of MK5 showed changes in anxiety-related behavior.

Applications

Compounds that specifically modify the phosphorylation of septin 8 by modulating the activity of MK5 or that interfere with the interaction between MK5 and septin 8 may have an impact on neuronal secretion and as such be used in patients suffering from perturbed neurosecretion.

Peer review

Overall, this is a potentially interesting paper; however, the data presented are preliminary.

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