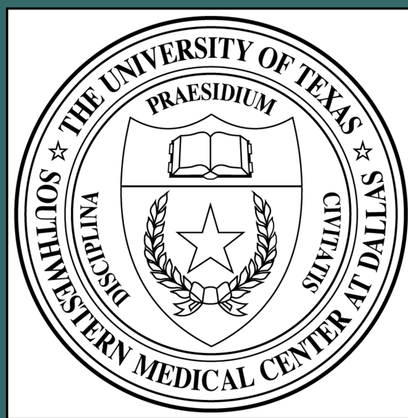

Cdk5-Dependent Regulation of Neuronal MEK1

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ABSTRACT

Cyclin-dependent protein kinase 5 (Cdk5) is a member of the Cdk family that is implicated in many regulatory pathways in post-mitotic neurons. The kinase plays an important role in neuronal development and synaptic transmission; its disregulation contributes to neuropathological diseases. Cdk5 is involved in the regulation of the Ras-Raf-MEK-ERK signaling pathway. It is hypothesized that Cdk5 serves a neuroprotective role by preventing the prolonged stimulation of ERK from inducing cell cycle reentry and, hence, neuronal apoptosis[1].

In order to regulation of MEK1 by Cdk5, we sought to confirm the Cdk5-phosphorylation site of MEK1. Although it has been previously shown that Cdk5 phosphorylates MEK1-T286[2], our mass spectometry analysis of *in vitro* kinase reactions identified the site as T292. We corroborated this data with Western blot analysis of the *in vitro* Cdk5 phosphorylation of MEK with phosphate-specific antibodies to sites T286 and T292. Thus we report a new target for the regulation of neuronal MEK1.

INTRODUCTION

The Ras/Raf/MEK/ERK signaling pathway integrates extracellular stimuli into intracellular responses that include transcription and cell cycle progression. The MEK1 catalytic domain contains the activation loop and Proline-Arginine Domain (PRD). The activation of MEK1 is dependent upon Raf interaction with the PRD and Raf phosphorylation of MEK1 at Ser218/Ser222 within its activation loop[3]. The ability of Raf to activate MEK is regulated by PAK phosphorylation of Ser298 within the PRD[4], and is potentially regulated by other kinase phosphorylation sites within the PRD. The kinase active during the G1/S-phase transition of the cell cycle, p34/cdc2, phosphorylates MEK1 at Thr286/Thr292 which has been shown to have an inhibitory effect[5]. The phosphorylation of Thr286 by Cdk5 has suggested an inhibitory effect, as well[2]. However, ERK phosphorylation of MEK at Thr292 has not shown any effect upon its kinase activity[6]. We sought to examine the role of Cdk5 phosphorylation of MEK1 and discovered a novel Cdk5 phosphorylation site, Thr292.

INTRODUCTION

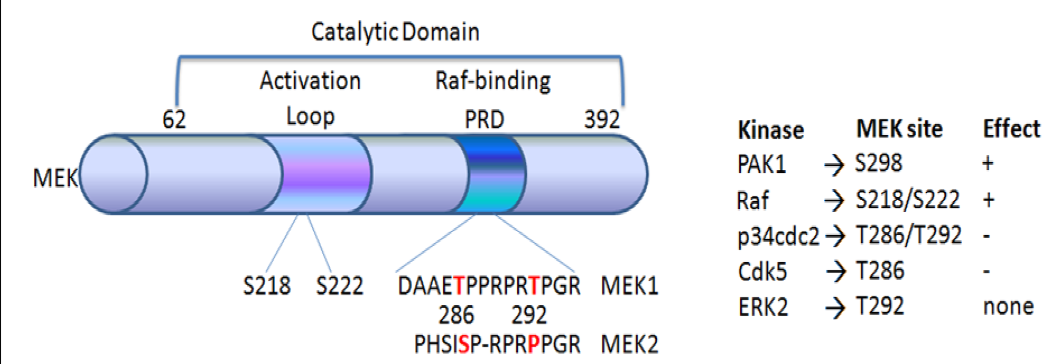

Figure 1. MEK1 Domains, MEK1/MEK2 sequence alignment and previously identified MEK1 phosphorylation sites. (Left panel) The catalytic domain spans amino acids 62-392, including the activation loop and PRD. (Lower panel) MEK2 does not show a consensus sequence similar to MEK1 at Thr286 and lacks a Thr292. (Right panel) Shown are the previously identified MEK1 phosphorylation sites with corresponding effects upon MEK1 activity. PAK1 phosphorylates at Ser298, Raf phosphorylates at Ser218/Ser222, p34/cdc2 phosphorylates at Thr286/Thr292, Cdk5 phosphorylates at Thr286, and ERK phosphorylates at Thr292.

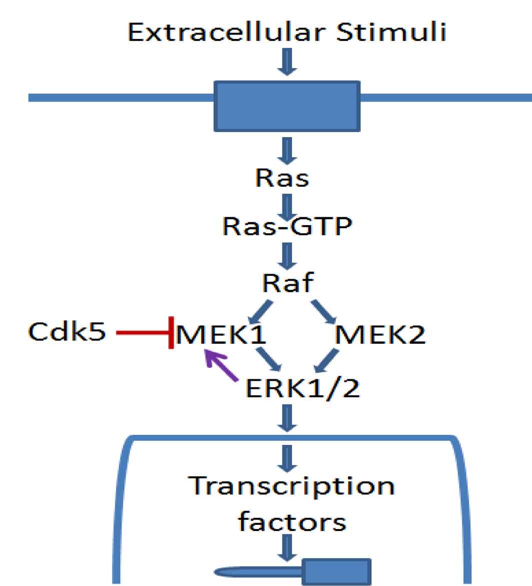

Figure 2. The MEK Pathway. Extracellular stimuli such as hormones, mitogens, vasoactive peptides, inflammatory cytokines of the tumor necrosis factor (TNF) family and environmental stresses activate the receptor tyrosine kinase. Consequently, this activates Ras, Raf, MEK, and ERK, concatenating in transcriptional activation. Cdk5 phosphorylates and may inhibit MEK activity, whereas, ERK phosphorylation at Thr292 has shown no effect.

METHODS

Purification of GST-MEK1

BL21 (DE3) *E. coli* cells were transformed with a bacterial expression vector with ampicillin resistance, pGEX, encoding GST-MEK1. Cells were plated in LB/ampicillin agar plates and single colonies were grown overnight in 100-mL LB/ampicillin. Overnight cultures, 10 mL, were added to 1L LB/ampicillin and grown at 37 °C until the OD600 reached 0.6-0.8 at which point 0.1 mg/ml isopropyl-b-D-thiogalactopyranoside (IPTG) was added. After overnight inductions at 25 °C, cells were harvested by centrifugation at 3000g for 30 min at 4 ° C. Cells were pelleted at 10,000*g* 15 min 4 ° C and were stored at –80 °C prior to lysis. Lysis was performed by freeze-thawing harvested samples and passing through a French press in 50 mM NaH_2PO_4 buffer, pH 8.0, containing 300 mM NaCl and protease inhibitors. Crude lysates were cleared by centrifugation at 10,000*g* for 30 min 4 ° C. Cleared lysates were incubated with glutathione agarose resin for one hour at 4 °C, followed by 5 washes in lysis buffer. Bound protein was eluted with a continuous gradient of 0–20mM glutathione, pH 8.0, in wash buffer. Samples were loaded into dialysis cassettes and dialyzed overnight in 10 mM Tris-HCl, pH 7.5, 1 mM DTT, through three changes of buffer (4 L each). Protein fractions were stored at –80°C following analysis for purity by SDS-PAGE and coomassie brilliant blue (CBB) staining. Protein concentrations were determined from the Bradford assay using BSA as a standard.

In vitro protein phosphorylation reactions

All *in vitro* protein phosphorylation reactions were performed at 30 ° C in a final volume of at least 30 ml containing 1.41mM MEK1, 100 mM ATP, and 0.2 mCi/ml [g-^{32}P]ATP, 30 mM HEPES, pH 7.2, and 5 mM MgCl2. Time-course reactions were performed by removing 10-ml aliquots from the reaction mixture at various time-points and adding 10-mL of 5X SDS protein sample buffer, followed by a 5-minute incubation at 80 ^{o}C to stop the reaction. In all cases, stoichiometries and enzyme activities were assessed by SDS-PAGE and PhosphorImager analysis for [^{32}P]phosphate incorporation. To calculate reaction stoichiometries, radiolabeled products and radioactive reaction standards were quantified by densitometry using ImageQuant software (Amersham Biosciences).

RESULTS

Bacterial Expression and Purification of GST-MEK1

Using the methods described in the previous section, an SDS-PAGE gel was run that included samples of elutions from column chormatography. The column chromatography aimed to separate out the MEK1 protein from the rest of the bacterial lysate sample. As shown in **Figure 3**, a purified sample of MEK1 bound to its GST tag was obtained. This enabled the progression to the subsequent parts of the experiment, where MEK1 had to serve as substrate to Cdk5, and allowed the phosphorylation site to be determined.

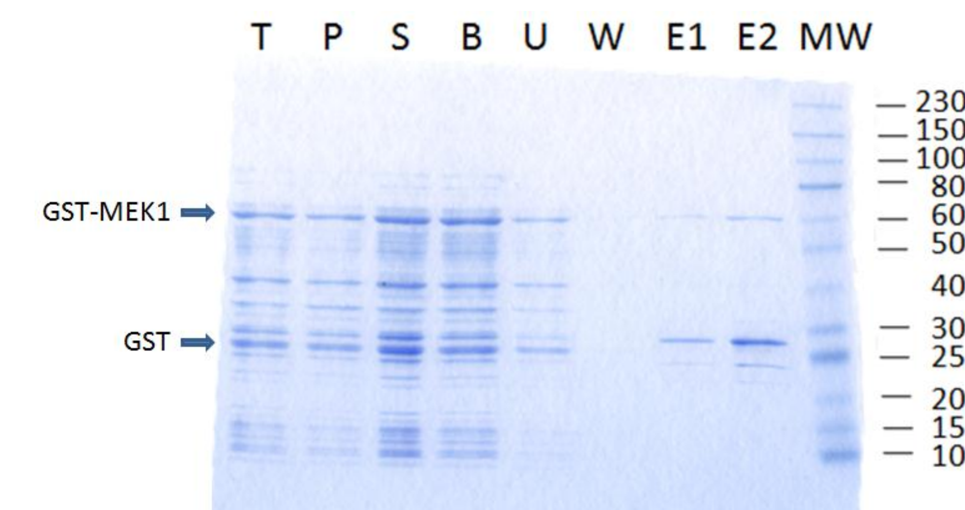

Figure 3. Bacterial Expression and Purification of GST-MEK1. The SDS-PAGE samples were stained with Coomassie Brilliant Blue. **(T)** total lysate, **(P)** pellet, **(S)** supernatant, **(B)** bound, **(U)** unbound, **(W)** wash, **(E1)** elution #1, **(E2)** elution #2, **(MW)** protein molecular weight marker, kDa.

Cdk5-dependent phosphorylation of MEK1

In order to explore the direct regulation of the ERK pathway by Cdk5, we tested MEK1 as a substrate for Cdk5 *in vitro*. MEK1 served as an efficient Cdk5 substrate *in vitro* (**Figure 4**). The phosphorylation of MEK1 by Cdk5/p25 yielded time-dependent incorporation of ^{32}P, reaching a final stoichiometry of approximately 0.65 mol/mol at 120 min.

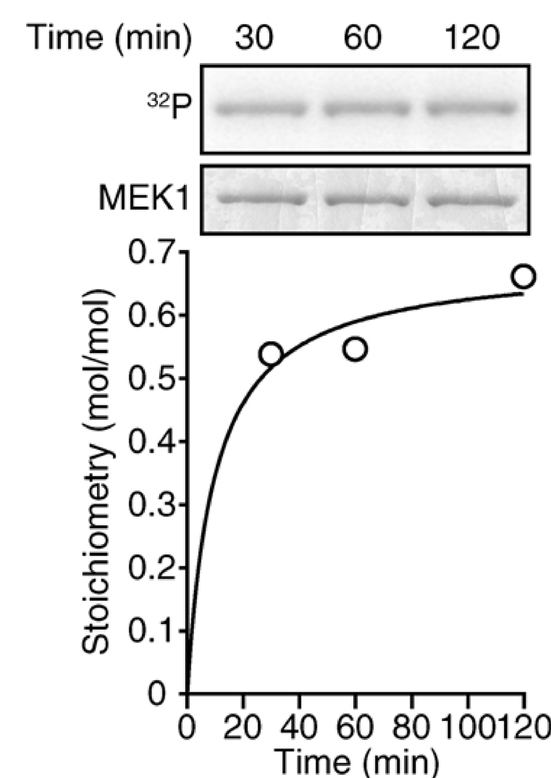

Figure 4. Evaluation of MEK1 as a substrate for Cdk5 in vitro. Evaluation of MEK1 as substrate for Cdk5-dependent phosphorylation. The panels show time course of *in vitro* phosphorylation of MEK1 by Cdk5/p25. Time-dependent incorporation of ^{32}P (top panel) and CBB-stained (bottom panel) MEK1 are depicted. Graph depicts stoichiometry of reaction at indicated times.

RESULTS

Identification of Thr292 as the site of Cdk5-dependent phosphorylation of MEK1

Previous reports have implicated Thr286 as the site of Cdk5-dependent phosphorylation of MEK. In order to determine the site of phosphorylation, mass spectrometry was conducted (data not shown) and yielded the result that a single site of phosphorylation must exist between residues 292 and 297, hence Thr292. In order to confirm this finding, we conducted Western blot analysis of samples from reactions in which MEK1 was phosphorylated by Cdk5/p25 (**Figure 5**). In these experiments, we detect a time-dependent increase in the level of pThr292 signal in the MEK1 product. There is a striking lack of signal in the immunoblot for pThr286 in the same samples. Western blot analysis with an antibody for total MEK1 shows equal loading of samples over the entire time course reaction. These data identify the phosphorylation of MEK1 by Cdk5 to be at Thr292, not Thr286 as previously published. These data will undoubtedly clarify the regulation of ERK signaling by Cdk5.

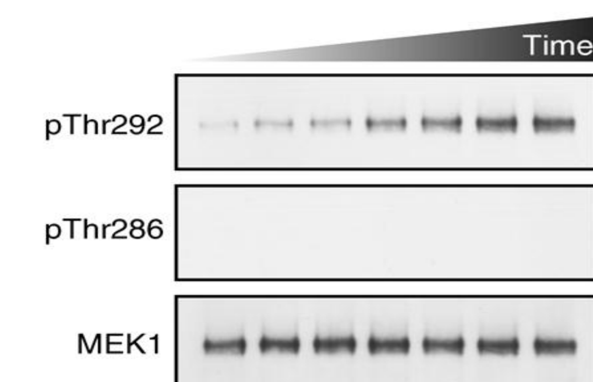

Figure 5. Confirmation of Thr292 as the site of Cdk5-dependent phosphorylation of MEK1. The panels show time course of *in vitro* phosphorylation (time course 0–180 min) of MEK1. Top panel depicts Western blot of reaction samples with phosphorylation state-specific antibody to pThr292 MEK1. Middle panel shows Western blot using pThr286 MEK1 antibody. Bottom panel depicts detection with total MEK1 antibody.

CONCLUSION

- Finally, the site of Cdk5-dependent phosphorylation of MEK1 was identified and confirmed as Thr292, rather than the previously reported Thr286.
- These data provide the foundation for further investigation into novel mechanisms by which Cdk5 may regulate the neuronal MEK/ERK signaling pathway.
- We intend to study the functional consequences of Cdk5 Thr292-phosphorylation upon MEK1 activity.

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