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ERK5 induces ankrd1 for catecholamine biosynthesis and homeostasis in adrenal medullary cells

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ABSTRACT

Extracellular signal-regulated kinases (ERKs) play important roles in proliferation, differentiation and gene expression. In our previous study, we demonstrated that both ERK5 and ERK1/2 were responsible for neurite outgrowth and tyrosine hydroxylase (TH) expression in rat pheochromocytoma cells (PC12) (*J Biol Chem* 284, 23,564–23,573, 2009). However, the functional differences between ERK5 and ERK1/2 signaling in neural differentiation remain unclear. In the present study, we show that ERK5, but not ERK1/2 regulates TH levels in rat sympathetic neurons. Furthermore, microarray analysis performed in PC12 cells using ERK5 and ERK1/2-specific inhibitors, identified ankyrin repeat domain 1 (ankrd1) as an ERK5-dependent and ERK1/2-independent gene. Here, we report a novel role of the ERK5/ankrd1 signaling in regulating TH levels and catecholamine biosynthesis. Ankrd1 mRNA was induced by nerve growth factor in time- and concentration-dependent manners. TH levels were reduced by ankrd1 knockdown with no changes in the mRNA levels, suggesting that ankrd1 was involved in stabilization of TH protein. Interestingly, ubiquitination of TH was enhanced and catecholamine biosynthesis was reduced by ankrd1 knockdown. Finally, we examined the relationship of ERK5 to TH levels in human adrenal pheochromocytomas. Whereas TH levels were correlated with ERK5 levels in normal adrenal medullas, ERK5 was down-regulated and TH was up-regulated in pheochromocytomas, indicating that TH levels are regulated by alternative mechanisms in tumors. Taken together, ERK5 signaling is required for catecholamine biosynthesis during neural differentiation, in part to induce ankrd1, and to maintain appropriate TH levels. This pathway is disrupted in pathological conditions.

1. Introduction

The mitogen-activated protein kinase family includes extracellular signal-regulated kinases (ERKs), ERKs are involved in proliferation, differentiation and gene expression. ERK1/2 are activated by variety of stimuli, and the signaling pathway leading to ERK1/2 activation has been well characterized [1–3]. The ERK5 kinase domain shares approximately 50% of homology with ERK1/2, but its unique long carboxy-terminus encodes two proline-rich regions, a nuclear export domain and a nuclear localization domain [4,5] and it plays a critical role in activating transcription [6]. The threonine and tyrosine residues on ERK5 are specifically phosphorylated by the upstream kinase, MEK5. ERK5 is also activated by growth factors, neurotrophic factors, cytokines and stressors, but the precise signaling pathways leading to ERK5 activation remain unclear. For example, involvement of small G proteins such as Ras and Rap1 in ERK5 activation remains controversial [7]. Ras-mediated ERK5 activation in rat pheochromocytoma cells (PC12 cells) and Rap1-mediated ERK5 activation in cortical neurons have been reported [8,9]. In contrast, we have shown that neither Ras nor Rap1 was required for ERK5 phosphorylation by nerve growth factor (NGF) or epidermal growth factor (EGF) in PC12 cells [10], whereas these small G-proteins are involved in the NGF-induced sustained ERK1/2 phosphorylation [11,12].

Abbreviations: ERK, extracellular signal-regulated kinase; PC12, pheochromocytoma cells; TH, tyrosine hydroxylase; ankrd1, ankyrin repeat domain 1; NGF, nerve growth factor; EGF, epidermal growth factor; bFGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; DMEM, Dulbecco's modified Eagle's medium; SCG, superior cervical ganglia; TBST, Tris-buffered saline containing 0.1% Tween-20; RT-PCR, reverse transcription-polymerase chain reaction; NLS, nuclear localizing signal; NES, nuclear export signal; TCF, ternary complex factors.

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The ERK5 gene knockout is lethal at E9.5–10.5 due to cardiovascular defects [13]. These defects resulted from abnormal vasculogenesis and angiogenesis in ERK5-lacking endothelial cells [14] rather than development of myocytes. Pathophysiological roles for ERK5 have been proposed for tumor development and cardiac hypertrophy [4,15]. There were no specific ERK5 inhibitors prior to the development of BIX02189 [16], and transfection efficiency in neuronal cells is low, so the approaches available to clarify the roles of ERK5 in neuronal cells have been limited. Genetic deletion studies showed that ERK5 is necessary and sufficient for neural differentiation of progenitor cells [17], and is critical for adult hippocampal neurogenesis regulating several forms of hippocampus-dependent memory formation [18,19]. We have shown that ERK5, along with ERK1/2, is essential for neurite outgrowth and expression of the neurotransmitter synthesizing enzyme, tyrosine hydroxylase (TH) in PC12 cells [10]. In rat C6 glioma cells, ERK5 and ERK1/2 were critical factors for gene expression of glial cell-derived neurotrophic factor [20]. Nevertheless, functional differences between ERK5 and ERK1/2, especially during the neural differentiation process remain unclear and require investigation.

In the present study, we report novel findings that ankyrin repeat domain 1 (ankrd1) is induced by ERK5, independent of ERK1/2, during neural differentiation and this ERK5-specific signaling molecule regulates TH levels and catecholamine biosynthesis. In addition, ERK5 regulation was observed in normal human adrenal medullas, but was disrupted in adrenal pheochromocytomas.

2. Materials and methods

2.1. Materials

NGF, basic fibroblast growth factor (bFGF), Hoechst-33258 and antibodies against neurofilament light chain, β-tubulin and β-actin were purchased from Sigma-Aldrich (St. Louis, MO). U0126, antibodies against phospho-ERK1/2, ERK1/2, phospho-ERK5, ERK5, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), flag (DYKDDDDK) and TH and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were purchased from Cell Signaling (Beverly, MA). This phospho-specific ERK5 antibody also recognizes phospho-ERK1/2. Antibodies against ERK2, ankrd1 and ubiquitin were purchased from Santa Cruz (Santa Cruz, CA). Enhanced chemiluminescence (ECL) assay kit, protein sepharose G and HRP-conjugated anti-mouse IgG were purchased from GE Healthcare (Buckinghamshire, England). Another ECL kit was purchased from PerkinElmer (Waltham, MA). Lipofectamine 2000, Neon electroporation kit, Alexa488-conjugated anti-rabbit IgG antibody and G418 were purchased from Invitrogen (Grand Island, NY). Non-immune rabbit immunoglobulin was purchased from Dako Japan (Tokyo, Japan). Growth factor-reduced Matrigel matrix was purchased from BD Biosciences (Franklin Lakes, NJ). MG132 was purchased from Peptide Institute (Osaka, Japan). TriPure isolation reagent (for total RNA extraction) was purchased from Roche (Indianapolis, IN). RT-PCR kit was purchased from Toyobo (Osaka, Japan). SYBR Premix Ex Taq (a kit for real-time PCR) was purchased from Takara (Otsu, Japan) or Roche. siRNA for rat ankrd1 was synthesized by B-Bridge (Mountain View, CA), and the cocktail of three duplexes was used, i.e. 1) sense 5′-GUU CAG AAA UGG AGA GUA UTT-3′ and antisense 5′-AUA CUC UCC AUU UCU GAA CTT-3′; 2) sense 5′-GAG CAU GCU UAG AAG GAC ATT-3′ and antisense 5′-UGU CCU UCU AAG CAU GCU CTT-3′; and 3) sense 5′-GAA UGG AAC CAA AGC GAU ATT-3′ and antisense 5′-UAU CGC UUU GGU UCC AUU CTT-3′. Rat ERK5 shRNA in pBAsi-mU6 vector (target Sequence 5′-CCA GCA ACT GTC CAA GTC T-3′) was created by Takara (Otsu, Japan). siRNA for rat ERK5 was synthesized by Invitrogen (sense 5′-CCG CGA UCU UAA ACC CUC UAA CCU U-3′ and antisense 5′-AAG GUU AGA GGG UUU AAG AUC GCG G-3′). DNA plasmid encoding flag-ankrd1 was kindly provided by Dr. Francesco Acquati (University of Insubria, Italy). BIX02189 was kindly provided by Boehringer Ingelheim (Ridgefield, CT).

2.2. Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Cell Culture Laboratory, Cleveland, OH), 5% horse serum (Invitrogen), penicillin (50 units/ml), and streptomycin (50 μg/ml) in an incubator containing 5% CO_2 at 37 °C. PC12 cells that stably overexpress ERK5 shRNA or flag-ankrd1 were cultured in the presence of G418. Sympathetic neurons were dissociated from the superior cervical ganglia (SCG) of newborn rat pups as described [21,22]. Both sexes of rat pups were used without regard to sex. Neurons were plated onto plates coated with poly-L-lysine and mouse type IV collagen. Cultures were maintained at 37 °C in 5% CO_2. Neurons were cultured in C2 media (DMEM/F12 1:1, BSA 0.5 mg/ml, L-glutamate 1.4 mM, selenium 30 nM, transferrin 10 μg/ml, insulin 10 μg/ml) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 50 ng/ml NGF, and 3% fetal bovine serum. Neurons were cultured with the anti-mitotic agent AraC (1 μM) for two days to deplete non-neuronal cells. Half the media was changed every two days, including new NGF. For transfection of sympathetic neurons, SCG were dissected from P3 Sprague Dawley rat pups. We used the Neon™ Transfection system and followed the manufacturer's instructions. Briefly, neurons were electroporated with specific parameters regarding pulse voltage (1500 V), pulse width (20 ms), and pulse number (1). Neurons were plated onto poly-L-lysine/collagen treated plates and grown in C2 media supplemented with NGF (50 ng/ml) and 3% fetal bovine serum. Neurons were grown in culture for 4 days before lysates were isolated and Western blotting was performed.

2.3. Assay for neurite outgrowth

The neurite extension from PC12 cells was regarded as an index of neuronal differentiation. The cells were fixed with 4% paraformaldehyde and the nuclei were stained with Hoechst-33258. The photographs were taken with CELAVIEW-RS100 (Olympus, Tokyo, Japan). The number of nuclei and total length of neurites were calculated with the CELAVIEW software (Olympus, Tokyo, Japan), then the value of total neurite length divided with nucleus number was expressed as a neurite length per cell (μm/cell). Data are expressed as means ± S.E.M. of the values of three wells [10]. To test the role of ERK5 in axon outgrowth, we used explants of SCG, which contain abundant sympathetic neurons. Ganglia were desheathed to facilitate axon outgrowth, embedded in reduced growth factor Matrigel matrix, and covered with serum-free DMEM/F12 with penicillin–streptomycin and 2 ng/ml NGF. Explants were maintained at 37 °C with 5% CO_2. A day after being plated, explants were photographed and incubated with or without U0126 and BIX02189, and then photographed again 6 h later. Axon length was measured using Image-J (Version 1.36b, National Institute of Health), and the rate of axon growth per hour was calculated as reported [23].

2.4. Microarray analysis

Total RNA from PC12 cells stimulated with NGF (100 ng/ml, 4 h) in the presence or absence of U0126 (30 μM) or BIX02189 (30 μM) was extracted using TriPure isolation reagent. Microarray slides (whole rat genome oligo microarray 4 × 44 k, Agilent Technology) were used for the transcript analysis. RNA labeling (Quick RNA amplification and labeling kit, Agilent Technology) and microarray hybridization (17 h at 65 °C) were carried out according to the supplied manual. The microarrays were washed after hybridization according to the Agilent manual and air-dried arrays were scanned with a 5 μm resolution in a high resolution Agilent microarray scanner. The scanned images were analyzed using GeneSpring GX7.3 (Agilent Technologies). Signal intensities for each probe were normalized to the 75th percentile. Genes that were up-regulated by more than three times by NGF were defined as NGF-stimulated genes. Among them, genes whose expression was inhibited