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MKK7γ1 reverses nerve growth factor signals: Proliferation and cell death instead of neuritogenesis and protection

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ABSTRACT

c-Jun N-terminal kinases (JNKs) are the exclusive downstream substrates of mitogen-activated protein kinase kinase 7 (MKK7). Recently, we have shown that a single MKK7 splice variant, MKK7γ1, substantially changes the functions of JNKs in naïve PC12 cells. Here we provide evidence that MKK7γ1 blocks NGF-mediated differentiation and sustains proliferation by interfering with the NGF-triggered differentiation programme at several levels: (i) down-regulation of the NGF receptors TrkA and p75; (ii) attenuation of the differentiation-promoting pathways ERK1/2 and AKT; (iii) increase of JNK1 and JNK2, especially the JNK2 54 kDa splice variants; (iv) repression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, which normally supports NGF-mediated cell cycle arrest; (v) strong induction of the cell cycle promoter CyclinD1, and (vi) profound changes of p53 functions. Moreover, MKK7γ1 substantially changes the responsiveness to stress. Whereas NGF differentiation protects PC12 cells against taxol-induced apoptosis, MKK7γ1 triggers an escape from cell cycle arrest and renders transfected cells sensitive to taxol-induced death. This stress response completely differs from naïve PC12 cells, where MKK7γ1 protects against taxol-induced cell death. These novel aspects on the regulation of JNK signalling emphasise the importance of MKK7γ1 in its ability to reverse basic cellular programmes by simply using JNKs as effectors. Furthermore, our results highlight the necessity for the cells to balance the expression of JNK activators to ensure precise intracellular processes.

1. Introduction

During neuronal differentiation, cells stop to proliferate and start to form neurites. This developmental programme is based on neutrophin binding to extracellular receptors, cellular internalisation of the signal, and followed by a crosstalk between different intracellular pathways (reviewed in [1,2]). The neutrophin nerve growth factor (NGF) activates the high affinity tyrosine receptor kinase A (TrkA) and the low affinity receptor p75. Activated TrkA recruites several adapter proteins and enzymes that ultimately propagate the NGF signal in the cell leading to differentiation. Predominantly, this happens through PI3K–AKT, Raf–MEK–ERK and MEKK1–MKK4/7–JNK signalling.

Whereas protein kinase B (AKT) and extracellular signal-regulated kinase 1/2 (ERK) signalling are mainly linked to differentiation and survival, the JNK pathway was often associated with neuronal death [3–7]. c-Jun N-terminal kinases (JNKs), however, are indispensible for neuronal differentiation and regeneration. Thus, *jnk1/jnk2* double knockout mice are embryonic lethal with an open neuronal tube and reduced cell death in the forebrain region [8,9]. *In vitro* studies with dominant-negative JNK isoforms and JNK inhibitors showed impairment of differentiation including NGF-induced neurite outgrowth and branching of PC12-N1 cells [10,11]. Exogenous expression of *jnk3* significantly increased NGF-induced neuritogenesis in PC12 cells, while the number of sprouting PC12 cells was decreased by expression of dominant-negative JNK2 [12,13]. However, the intracellular signalling that regulates JNK functions of neurite formation and apoptosis is still poorly understood.

MKK4 and MKK7 are the direct activators of JNKs [14–16]. Whereas MKK4 additionally activates p38 mitogen-activated protein kinases (MAPK), MKK7 exclusively phosphorylates JNKs [17–19]. So far, six murine, four human and two rat MKK7 splice variants encoded by a single gene, have been described. These splice variants differ in their N-termini (α, β and γ isoforms) and C-termini (1 and 2 isoforms) [19–23]. MKK7β and MKK7γ display higher basal activities than MKK7α presumably caused by more JNK-binding sites [24]. Unfortunately, *mkk7* knockout is embryonic lethal making functional *in vivo* studies impossible [25]. Studies on $mkk7^{-/-}$ mouse embryonic fibroblasts and mast cells revealed conflicting data, but suggested important roles for MKK7 in the regulation of proliferation [25,26]. However, splice variant-specific studies are still missing. We have

Abbreviations: AKT, protein kinase B; ER, endoplasmatic reticulum; ERK, extracellular signal-regulated kinase; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK-1, mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase 1; MEKK-1, mitogen-activated protein kinase/Erk kinase kinase-1; MKK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; p21, $p21^{WAF1/CIP1}$; PI3K, phosphatidylinositol-3-kinase; TrkA, tyrosine receptor kinase A.

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recently shown that MKK7γ1 overexpression has an anti-proliferative and pro-apoptotic effect in mitotic PC12 cells under normal cell growth conditions, while cellular stress induces a pro-proliferative and anti-apoptotic MKK7γ1 signalling [20]. Both MKK7γ1-mediated effects triggered a distinct activation of JNK signalling. These observations on highly specific MKK7γ1 functions raised the question whether MKK7γ1 also regulates the differentiation of PC12 cells by down-regulation of cellular proliferation.

In the present study, we have investigated the role of MKK7γ1 in neuronal differentiation of rat pheochromocytoma PC12 cells stably transfected with MKK7γ1. PC12 cells respond to NGF by the induction of growth arrest and the formation of a neuronal phenotype [27]. Overexpression of MKK7γ1, however, prevented complete differentiation of PC12 cells by promoting proliferation. ERK1/2 and AKT were down-regulated, accompanied by an up-regulation of specific JNK isoforms and the differential expression and activity patterns of JNK targets involved in proliferation and differentiation. Finally, the pro-proliferative effect of MKK7γ1 made PC12 cells susceptible to taxol-induced apoptosis during NGF supplementation. This study impressively demonstrates that a single JNK activator is capable to reverse cellular programmes dependent on the (patho-)physiological context. Apparently, JNKs are executors of MKK7 signalling and the understanding of JNK signalling requires a novel focus on MKKs.

2. Materials and methods

2.1. Cell culture

Rat pheochromocytoma PC12 cells (ATCC/LGC Promochem) were cultured on collagen-coated plates in RPMI 1640 (PAA) supplemented with 5% foetal bovine serum (Lonza) and 10% horse serum (Invitrogen) at 37 °C and 5% CO_2. Transfected cell clones were kept in the presence of 500 μg/ml G418 (Biochrom) to maintain selection. For differentiation, PC12 cells were kept in growth medium for 24 h, then in medium supplemented with 0.5% FBS and 1% penicillin/streptomycin for 72 h to synchronise the cell cycle activity, before adding recombinant mouse 2.5 S NGF (50 ng/ml; Alomone Labs) for 5 days.

2.2. Plasmids and transfection

cDNA of MKK7γ1 (GenBank™ accession number ID: GU264001) from PC12 cells was inserted in the expression vector pEGFP-C3 (Clontech) as a *BamH*I/*Hind*III fragment. Transfection of the MKK7γ1–pEGFP construct or the pEGFP vector was performed using the Turbofect™ transfection system (Fermentas) according to the manufacturer's instructions. Individual stable clones that expressed the MKK7γ1–pEGFP construct or the pEGFP vector were selected and used for experiments. Microscopy, cell counts and Western blots with five independent stable PC12 clones revealed the same results.

2.3. Cell counting

For trypan blue staining, 20 μl of the cell suspension was mixed with 20 μl of trypan blue solution and transferred to a hemocytometer twin chamber (Omnilab). For reasons of accuracy, the cells were not allowed to stay in the dye solution for longer than 5 min. Living cells in the 16 squares of both chambers were counted, and the percentage of viable cells was determined.

2.4. Neurite outgrowth

For examination of neurite outgrowth, 1×10^5 vector- and MKK7γ1-transfected PC12 cells were grown on an object slide and differentiated for 5 days with 50 ng/ml NGF. The percentage of cells with neurites longer than 1.5 diameters of the cell body was counted using the software ImageJ.

2.5. Whole cell extracts and nuclear extracts

If not indicated otherwise, chemicals were purchased from Sigma–Aldrich or Carl Roth. Whole cell lysates were generated from subconfluent cells. Before harvesting, the cells were washed with phosphate-buffered saline. For whole cell extracts, the cells were resuspended in denaturing lysis buffer (20 mM Tris (pH 7.4), 2% SDS, 1% phosphatase inhibitor, and 1% protease inhibitor), incubated at 95 °C for 5 min, briefly sonicated, and centrifuged to remove insoluble material (15,000×*g* for 15 min). To obtain nuclear and cytoplasmic extracts, the cells were lysed as described previously [28]. Protein extracts were stored at −80 °C. The protein concentration of the supernatant was determined by the Bio-Rad protein assay, with bovine serum albumin as standard. The purity of the nuclear and cytoplasmic fractions was tested by Western blot with an antibody directed against activating transcription factor-2 (ATF-2).

2.6. Western Blot

Twenty micrograms of the total cellular proteins were separated on 12% SDS-polyacrylamide gels and transferred to poly-vinylidene difluoride transfer membranes (Millipore). The membranes were blocked with 4% non-fat dry milk and incubated with the primary antibodies according to the manufacturer's recommendations. After three washing steps with TBST, the membranes were incubated with the HRP-conjugated secondary antibody for 30 min. All Western blots were developed using the ECL chemiluminescence system and Hyperfilm ECL (GE Healthcare). Between the stainings with phosphospecific antibodies and total kinase or total transcription factor antibodies, blots were stripped in 2% SDS, 62.5 mM Tris and 100 mM 2-mercaptoethanol for 30 min at 50 °C, washed with TBST and blocked again. All measurements of dual-phosphorylated kinase levels were normalised by hybridization with antibodies against the total kinase protein. To normalise for the protein content of each lane and to confirm equal loading, all membranes were finally stained with Ponceau S. Antibodies against the following targets were purchased from the indicated sources: anti-mouse IgG (GE Healthcare); phospho-AKT, phospho-JNK and phopho-p38 (Promega); Akt, cleaved Caspase-3, c-Myc, JNK, MKK7, p53 , phospho-c-Jun, phospho-c-Myc, phospho-ERK1/2, phospho-MKK4, phospho-MKK7, phospho-p53 and anti-rabbit IgG (Cell Signaling); CyclinD1 and JNK1 (BD Pharmingen); Caspase-3, JNK2, MEK-1, MEKK-1, MKK4, p21/WAF1, p38, p75, phospho-MEK-1, and TrkA (Santa Cruz).

2.7. Immunoprecipitation

For immunoprecipitation (IP), native cell lysates were prepared from subconfluent cells. Before harvesting, the cells were washed with phosphate-buffered saline. For whole cell extracts, the cells were resuspended in native lysis buffer (20 mM Tris (pH 7.6), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0,5% Non-Idet P40, 1% phosphatase inhibitor, 1% β-glycerolphosphate and 1% protease inhibitor), incubated on ice for 30 min and centrifuged to remove insoluble material (15,000×*g* for 15 min). One hundred fifty μg of the total proteins were used for IP with the ExactaCruz™ reagents (Santa Cruz). All steps were performed at 4 °C if not mentioned otherwise. The samples were precleared for 30 min with the appropriate preclearing Matrix. The antibody-IP matrix complex was formed by incubation of 1 μg primary antibody with 50 μl of the appropriate IP matrix in 500 μl of PBS for 1 h. The IP antibody-IP matrix complex was washed twice with cold PBS, the pre-cleared sample was mixed with the antibody-IP matrix complex and incubated overnight. The negative control was prepared by incubation of the antibody-IP matrix complex with native lysis buffer. After centrifugation, the pellets containing immobilised proteins with IP matrix complexes were washed three times with PBS. The immobilised proteins were dissociated with 5× reducing