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The role of Src protein in the process formation of PC12 cells induced by the proteasome inhibitor MG-132

Oktávia Tarjányi [a,b], Gergely Berta [a,b], Alexandra Harci [a,b], Eszter B. Bacsa [a], Borbála Stark [a], Marianna Pap [a,b], József Szeberényi [a,b], György Sétáló Jr. [a,b,*]

[a] Department of Medical Biology, Medical School, University of Pécs, Pécs, Hungary, Szigeti út 12, H-7643 Pécs, Hungary
[b] Signal Transduction Research Group, János Szentágothai Research Centre, Pécs, Hungary, Ifjúság útja 20, H-7624 Pécs, Hungary

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ABSTRACT

The PC12 (rat pheochromocytoma) cell line is a popular model system to study neuronal differentiation. Upon prolonged nerve growth factor (NGF) exposure these tumor cells stop to divide, become polygonal, grow projections and start to look and behave like sympathetic neurons. Differentiation of PC12 cells can also be induced by peptidyl-aldehyde proteasome inhibitors, such as Z-Leu-Leu-Leu-al (also known as MG-132) or via infection of the cells with Rous sarcoma virus. The signal transduction pathways underlying process formation, however, are still not fully understood. The liganded NGF receptor initiates a protein kinase cascade a member of which is Extracellular Signal-Regulated Kinase (ERK). Active ERK1/2 enzymes phosphorylate various cytoplasmic proteins and can also be translocated into the nucleus, where they regulate gene expression by activating key transcription factors. Using immunological methods we detected phosphorylation of TrkA, prolongedactivation of Src, and ERK1/2 with nuclear translocation of the latter during MG-132-induced process formation of PC12 cells. Activated Src remained predominantly cytoplasmic. MG-132-induced sustained ERK1/2 activation, nuclear translocation and neuritogenesis required the intact function of Src since these phenomena were markedly reduced or failed upon chemical inhibition of Src tyrosine protein kinase activity.

1. Introduction

PC12 cells are derived from a tumor of the rat's adrenal medulla. Upon prolonged exposure to nerve growth factor (NGF) these cells stop to proliferate, grow in size and develop processes, becoming similar to sympathetic neurons both in appearance and behavior (Greene and Tischler, 1976). Complex signaling events are initiated behind these phenomena by the binding of NGF to its high affinity receptor, tropomyosin-related kinase (TrkA) (Chao and Hempstead, 1995; Esposito et al., 2001). Activation of the monomeric G-protein Ras is then followed by the induction of a cytoplasmic protein kinase cascade, with MEK (mitogen-activated proteinkinase/ERK kinase) and ERK1/2 (extracellular signal-regulated kinase 1 and 2) being its main downstream effectors (Segal and Greenberg, 1996; Szeberényi and Erhardt, 1994; Vaudry et al., 2002). Prolonged activation and nuclear translocation of ERK 1/2 is a necessary requirement of neuronal differentiation in PC12 cells, whereby nuclear ERK can alter gene expression through phosphorylation of key transcription factors (Boglári et al., 1998; Marshall, 1995; Traverse et al., 1992).

Additional pathways can also play a role in PC12 cell differentiation (Szeberényi, 1991). One of these is mediated by the tyrosine protein kinase Src. Expression of the v-src oncogene's protein product in PC12 cells induces neurite outgrowth similar to that resulting from NGF treatment (Alemà et al., 1985; Rausch et al., 1989; Thomas et al., 1991). In the active form Src is not phosphorylated on its C-terminal tyrosine, another tyrosine in the catalytic domain

Abbreviations: Cy3, a fluorophor cyanin dye; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl-sulfoxyde; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ERK1/2, extracellular signal-regulated kinase 1 and 2; H-Ras, an isoform of the small guanine nucleotide-binding protein Ras named after Harvey; IκB, inhibitor of kappa B; JNK, c-Jun N-terminal kinase; MAP Kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/ERK kinase; MG-132, a peptidyl-aldehyde proteasome inhibitor, also known as Z-Leu-Leu-Leu-al; M-M17-26, a variant PC12 clone that stably expresses a dominant negative H-Ras mutant; NGF, nerve growth factor; nnr5, a TrkA-deficient PC12 variant clone; p38MAPK, a MAP kinase with the molecular weight of 38 kDa; PBS, phosphate buffered saline; PC12, rat pheochromocytoma cell line; PD98059, a chemical compound that inhibits MEK; PMSF, phenylmethanesulfonyl fluoride; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, an inhibitor of Src; PVDF, polyvinylidene difluoride; Ras, a monomeric G-protein; Src, a non-receptor tyrosine kinase; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TrkA, tropomyosin-related kinase.

* Corresponding author at: Department of Medical Biology, Medical School, University of Pécs, Pécs, Hungary, Szigeti út 12, H-7643 Pécs, Hungary. Tel.: +36 72 536 216; fax: +36 72 536 453.
E-mail addresses: gyorgy.setalo.jr@aok.pte.hu, setalogy@citromail.hu (G. Sétáló Jr.).

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will, however, be at the same time maximally phosphorylated (Smart et al., 1981).

The ubiquitin–proteasome system plays an important role in the degradation of proteins regulating the cell cycle, apoptosis (Hattori et al., 1999) and cellular metabolism (Doherty et al., 2002; Pickart, 2001). NGF-induced neurite outgrowth coincides with elevated levels of endogenous ubiquitin and ubiquitinated proteins (Obin et al., 1999). Various signal transduction molecules, among them Src can become the victim of proteasome-mediated degradation as the active form of this kinase is broken down through the ubiquitin–proteasome pathway (Hakak and Martin, 1999). The role of the proteasome in protein degradation is well established (Hershko and Ciechanover, 1998; Verma et al., 2004).In addition, the proteasome has been implicated in a number of biological processes, like receptor internalization, protein sorting, subnuclear trafficking, gene expression or neuronal differentiation (Pickart, 2001; Rubinsztein, 2006).

Proteasome inhibitors have been shown to induce neurite outgrowth (Fenteany et al., 1994; Inoue et al., 2004; MacInnis and Campenot, 2005; Momose et al., 2002; Obin et al., 1999) and enhance the differentiation of oligodendroglial cells (Pasquini et al., 2003). MG-132 is a peptidyl-aldehide, peptidomimetic type proteasome inhibitor (Lee and Goldberg, 1998). Consequently it is in a position to reduce the degradation of various signaling proteins, including that of active Src.

In this study, we investigated the possible role and intracellular localization of Src and ERK1/2, well known components of the differentiative signaling of PC12 cells. Our attention was focused onto the involvement of Src in the MG-132-induced differentiation processes of PC12 cells as we chemically inhibited the negative regulatory pathway of Src mediated by the proteasome or, alternatively, we blocked the activity of Src itself.

2. Materials and methods

2.1. Reagents

All chemicals used were from Sigma–Aldrich (Budapest, Hungary) unless otherwise stated. NGF and the Src inhibitor PP2 were purchased from Invitrogen (Paisley, Scotland, UK). The peptidyl-aldehyde proteasome inhibitor MG-132 has been selected based on its relatively good membrane permeability (Saito and Kawashima, 1989; Saito et al., 1990) and the studies of Giasson et al. (1999) analyzing the stress inducing effect of this compound. Safe concentration (2.5 μM) and treatment times to avoid possible toxic effects were determined based on the schemes of Song et al. (2009) and Song and Yoo (2011) and were further supported by healthy cell and nuclear morphology during our experiments, also documented on confocal images using fluorescence labeling or a combination of it withphase contrast detection (for examples see Fig. 9). Pilot experiments repeatedly confirmed that dimethyl-sulfoxyde (DMSO), the vehicle of non-water-soluble chemicals had no effect on the examined parameters of the experiments at the concentrations applied (0.025 v/v % for single and 0.05 v/v % for combined treatments, not shown).

2.2. Tissue culture

PC12 rat pheochromocytoma cells and their variant clones (M-M17-26 that stably expresses a dominant negative H-Ras mutant or the TrkA deficient nnr5 cells, all three PC12 cell lines kindly provided by G.M. Cooper) passage number 5–15 were plated onto plastic Petri dishes or Thermanox (Nalgene Nunc International, Rochester, NY, USA) coverslips (at 50% or 30% confluence, respectively) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) complemented with 5% fetal bovine and 10% horse serum, for a day to achieve sufficient adhesion. The cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2. Next day the media were replaced by 0.5% horse serum-containing DMEM for 24 h to silence serum-stimulated pathways before and during treatments.

2.3. Western blotting

Upon completion of the treatments the cultures were scraped into ice-cold lysis buffer (50 mM Tris-base, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM Na-orthovanadate, 5 μM $ZnCl_2$, 100 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1% Triton X-100) and stored frozen. On the day of processing samples were thawed and homogenized by vortexing for 20 s. The homogenates were centrifuged at 40,000×*g* at 4 °C for 30 min and the protein concentration of the supernatants was determined (Lowry's method, Detergent Compatible Protein Assay Kit, Bio-Rad, Hercules, CA, USA). Samples containing equal amounts of protein (50 μg in case of Src/p-Src or TrkA/p-TrkA and 25 μg for therest) were mixed with 4× Laemmli buffer (25 ml 1 M Tris–HCl, pH 6.8, 40 ml glycerol, 8 g SDS, 10 ml 100 mM EDTA, 10 ml 100 mM EGTA and 1 ml 1% bromophenol blue brought up to 100 ml with distilled water), and boiled for three minutes. Subsequently, they were loaded onto 10% polyacrylamide gels to perform size-based separation. The gels were electro-blotted onto PVDF membranes (Hybond-P, GE Healthcare, UK).

Immunodetection of the protein of interest was carried out by blocking the membrane in 3% nonfat dry milk dissolved in TBS-Tween (10 mM Tris-base, 150 mM NaCl, 0.2% Tween-20, pH 8.0), followed by addition of the primary antibody [anti-TrkA (R&D Systems, Minneapolis, MN, USA), anti-β-actin, phospho-TrkA and phospho-p44/42 MAP Kinase to detect phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA, USA), ERK-1 (C-16), ERK-2 (C-14) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphospecific (Tyr 418) polyclonal anti-Src (Biosource, Camarillo, CA, USA), monoclonal anti-active Src recognizing Src that is phosphorylated at Tyr 418 but unphosphorylated at Tyr 529 (Invitrogen)] diluted in the blocking solution 1:1000 and incubated overnight. Methodical control samples prepared by the omission of the primary antibodies produced no immune signal as determined in pilot experiments. (The reliability of TrkA and phospho-TrkA antiserahas also been confirmed by the lack of specific signals in lysates of the TrkA-deficient PC12 variant nnr5 cells.) Excess antibody was removed by five washes of TBS-Tween. Membranes were incubated with a horseradish-peroxidase (HRP)-conjugated secondary antibody (Pierce, Thermo Fischer Scientific, Rockford, IL, USA) diluted 1:50,000 in blocking solution. Five washes in TBS-Tween were followed by detection of the enhanced chemiluminescent signal (Immobilon Western, Millipore Corporation, Billerica, MA, USA). Densitometry was carried out using the ImageJ software (National Institutes of Health, USA).

2.4. Data presentation of Western blots

Representative photos are from series with similar results. Densitometric values are the mean ± S.D. for the indicated repeat number of independent experiments. Significance of differences was determined using ANOVA testing applying Bonferroni corrections for multiple samples. *P* values <0.05 were considered to be significant. Significant differences relevant to major findings are marked in the graphs and their corresponding *P* values are indicated in the figure legend.

2.5. Fluorescence microscopy

Treatments were stopped by rinsing in 37 °C PBS (1.37 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4{\cdot}7H_2O$, 0.14 mM KH_2PO_4,