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NUCLEAR TRANSLOCATION OF $p90^{Rsk}$ AND PHOSPHORYLATION OF CREB IS INDUCED BY IONOMYCIN IN A RAS-INDEPENDENT MANNER IN PC12 CELLS

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In the present study we examined the possible role of $p90^{Rsk}$ in pathways leading to neuronal differentiation of PC12 cells induced by nerve growth factor (NGF) and the calcium ionophore ionomycin. PC12-M17 cells, expressing a dominant inhibitory Ras protein, do not undergo neuronal differentiation in response to NGF like wild-type PC12 cells, but exhibit neurite outgrowth when treated with NGF in combination with ionomycin. However, the blockade of Ras in these cells results in failure of activation of mitogen-activated protein kinase (MAPK)/extracellular signal regulation kinase (ERK) (MEK) and ERK activation as well, therefore kinases other than those of the ERK pathway might play a role in the induction of neuronal differentiation in this case. Here we show that $p90^{Rsk}$ translocates to the nucleus in response to ionomycin in both wild-type PC12 and PC12-M17 cells, and this spatial distribution is followed by increased phosphorylation of the cAMP response element binding protein (CREB). Since CREB is believed to be the transcription factor that can integrate Ca^{2+}, growth factor and cAMP-induced signals, we suggest that $p90^{Rsk}$ may be one of the kinases which is able to replace ERKs under certain circumstances, thereby participating in Ras-independent neuronal differentiation induced by NGF plus ionomycin.

Keywords: PC12 cells – neuronal differentiation – Ras – $p90^{Rsk}$ – CREB

INTRODUCTION

The PC12 cell line established from a rat pheochromocytoma [14] is the most widely used model system to study the molecular background of neuronal differentiation. These cells exhibit sympathetic neurite outgrowth upon nerve growth factor (NGF) treatment, whereas undergo proliferation in the presence of epidermal growth factor (EGF) [19]. The main function of NGF is to regulate the survival, growth and differentiation of certain sensory and sympathetic neurons. Upon binding of its ligand, the $p140^{Trk}$ NGF receptor becomes autophosphorylated and activated, recruiting adaptor proteins to the plasma membrane. The signal is then relayed to the monomeric guanine-nucleotide binding Ras protein, which is activated by exchanging its GDP for GTP and passes the signal to downstream target proteins [29]. Among the possi-

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ble effectors, a protein kinase cascade is thought to be the main cytoplasmic pathway in NGF signaling, including Raf kinase, MEK (MAPK/ERK kinase) enzymes and the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein kinases (MAPKs). ERKs and their downsteam effector, p90 ribosomal S6 kinase ($p90^{Rsk}$) are believed to be directly involved in the regulation of gene expression by phosphorylating specific transcription factors in the nucleus [2, 33]. Although several publications provide evidence for the significance of ERK activation and nuclear translocation in the differentiation pathway [13, 19, 23, 31], the precise role of $p90^{Rsk}$ in differentiation signaling has yet to be elucidated. Nevertheless, $p90^{Rsk}$ has been shown to be the major Ca^{2+}-stimulated CREB kinase, phosphorylating the cAMP response element binding protein (CREB) at its critical regulatory site [35]. In addition, $p90^{Rsk}$ is able to translocate to the nucleus upon depolarization [18], as well as in response to NGF together with the ERK enzymes in PC12 cells [23]. The involvement of $p90^{Rsk}$ in the neuronal differentiation of PC12 cells, and the role of Ras protein in it, can be conveniently studied by using subclones of PC12 cells in which the endogenous Ras activity is blocked by a dominant interfering mutant Ras (Ha-Ras Asn-17) protein. Such mutant PC12 transfectants (called M17 subclones) expressing the dominant inhibitory Ras protein at different levels have already been isolated and characterized [27, 28]. For instance, in the M-M17-26 subclone (referred to as PC12-M17 cell line in this paper), expressing the mutant Ras at a high level, the NGF-induced differentiation response, as well as the induction of several early-response genes (such as c-*fos*, c-*jun*, *zif* 268, *jun*B) are blocked [27]. However, these cells are able to exhibit neurite outgrowth when treated with NGF in combination with ionomycin, a Ca^{2+} ionophore or dbcAMP [28], and this differentiation was shown to be independent of the classic Ras/MEK/ERK pathway (Boglári and Szeberényi, in press). Since the convergence point at which Ca^{2+}, cAMP and NGF-stimulated pathways may cross-talk in the signaling events has yet to be identified, in this paper we tested the possibility of the involvement of $p90^{Rsk}$ in the ionomycin-stimulated pathway. In order to analyze the spatial distribution of $p90^{Rsk}$ we performed immunocytochemical staining in wild-type PC12 as well as PC12-M17 cells in response to NGF and ionomycin. In addition, by using an anti-phospho-CREB antibody in a Western blot protocol we examined CREB phosphorylation in both cell lines upon the same treatments. Our results show that translocation of $p90^{Rsk}$ can be induced by both NGF and ionomycin in wild-type PC12 cells, however, the inhibition of Ras in PC12-M17 cells blocks the NGF-induced redistribution of the enzyme, but it is not affected in response to ionomycin. In accordance with these results, CREB phosphorylation was detected in both cell lines upon ionomycin treatment, but was blocked in response to NGF in the PC12-M17 cells. These data suggest a possible role of $p90^{Rsk}$ in a Ras independent, Ca^{2+}-stimulated pathway contributing to neurite formation without the involvement of the classic Ras/MEK/ERK route in PC12 cells.

MATERIALS AND METHODS

Cell culture

Wild-type PC12 and PC12-M17 cells were grown on 100 mm tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% horse and 5% fetal bovine serum and maintained in a humidified incubator with 5% CO_2 at 37 °C. Treatment of cells was carried out with NGF at a concentration of 50 ng/ml or ionomycin (0.25 μM) from 5 to 30 min in low serum containing (0.5% horse serum) medium.

Immunocytochemistry

Cells were plated at 10^6 cells per 100 mm dish and grown for 1 day in serum-containing medium. Afterwards, 2 days of total serum starvation was carried out and cells were treated with NGF or ionomycin as described above. Cells were collected and subjected to centrifugation in Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 500 rpm for 5 min in order to spread them onto microscopic slides at 5×10^4 cells per slide. Cells were dried onto the slides for 1 day then fixed with ice-cold acetone for 10 min and washed with TBS three times for 5 min. Blocking of possible endogenous peroxidase activity was performed in TBS containing 1% H_2O_2 for 10 min, then cells were washed with TBS three times for 5 min. Non-specific binding of antibodies was blocked with 1% bovine serum albumin (BSA) in TBS for 20 min. Cells were then incubated with the primary antibody in the appropriate dilution in 1% BSA (p90^{Rsk} 1 : 1000, Santa Cruz Biotech, Santa Cruz, California, USA) for 1 hour. After washing the cells as described above, they were incubated with the secondary antibody (horseradish peroxidase /HRP/ conjugated to anti-rabbit gamma globulin in 1 : 100 dilution, Santa Cruz Biotech) for 30 min. After the washing procedure the immunological binding reaction was detected by using a Ni-DAB (diamino-benzidine) reagent. Cells were then mounted with DePex without counterstaining.

Phospho-CREB Western blotting

Cells were plated at 5×10^6 cells per 100 mm dish and grown for one day in serum-containing and one day in serum-free medium (to decrease base level CREB phosphorylation) before treatment. Treatments were carried out as described above for the desired times, then protein isolation was performed following the Santa Cruz (California, USA) protocol. The total cell lysate was then subjected to SDS polyacrylamide gel electrophoresis followed by electroblotting of the separated proteins onto a nitrocellulose filter. Detection of phosphorylated CREB was performed as follows: rinsing the membrane in 5% skimmed milk/PBS for 1 hour, incubation with anti-phospho-CREB antibody (1 : 500, New England Biolabs GmbH, Frankfurt am Main, Germany) for 1 hour, washing 3×5 min with 1% milk/PBS, incubation with

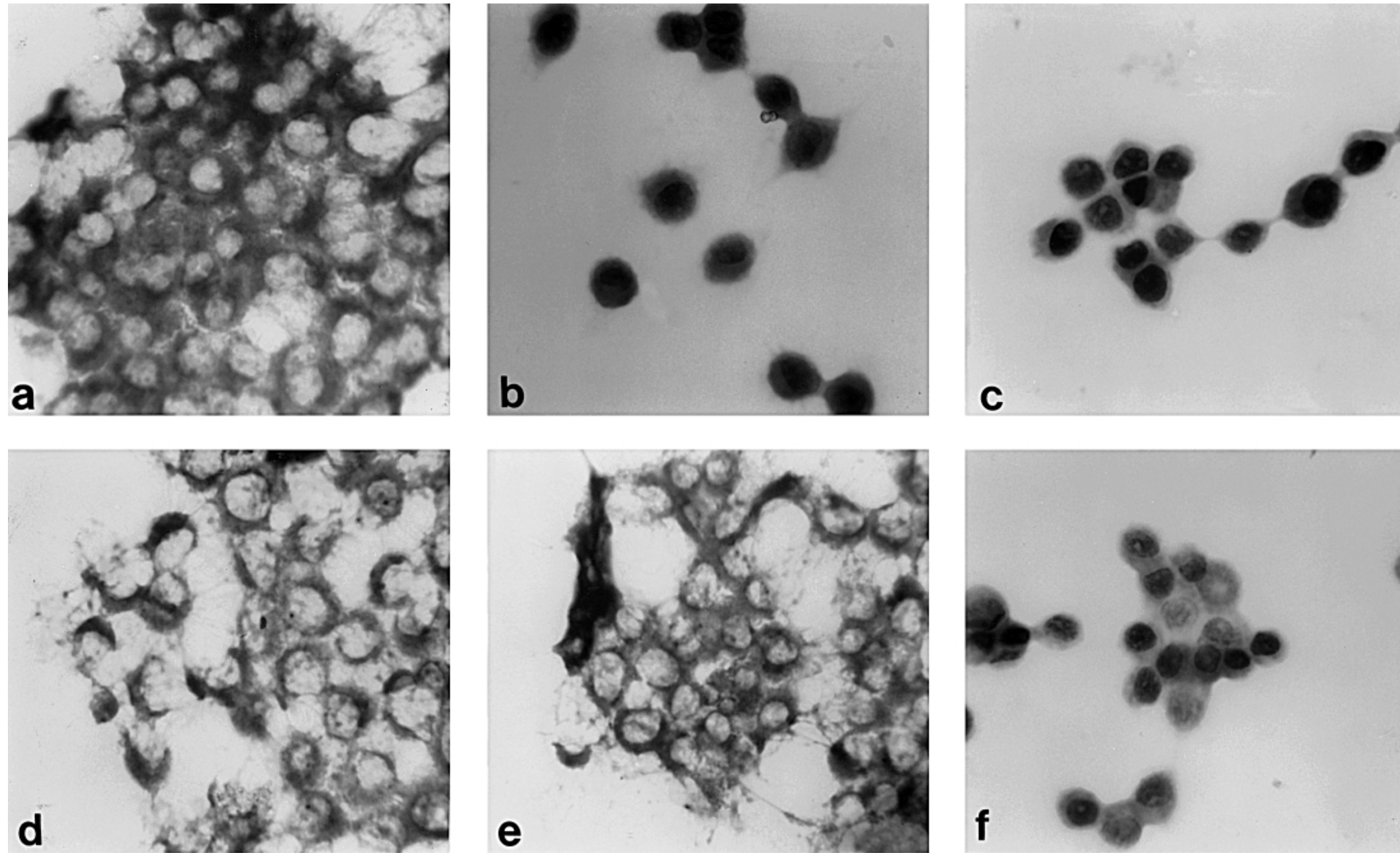

Fig. 1. Immunolocalization of p90^{Rsk} in PC12 subclones in response to various treatments. a – untreated wild-type PC12 cells; b – wtPC12 cells treated with NGF (50 ng/ml) for 30 min; c – wtPC12 cells treated with ionomycin (0.25 µM) for 30 min; d – untreated PC12-M17 cells; e – PC12-M17 cells treated with NGF (50 ng/ml) for 30 min; f – PC12-M17 cells treated with ionomycin (0.25 µM) for 30 min

the secondary antibody (HRP-conjugated anti rabbit Ig, 1 : 2000, New England Biolabs, Germany) for 30 min, washing 3×20 min and then detection of the chemiluminescence signal by using the ECL kit (Amersham Pharmacia Biotech Export GmbH, Vienna, Austria).

RESULTS

Nuclear translocation of p90^{Rsk}

In order to analyze the effect of NGF and ionomycin on the nuclear translocation of p90^{Rsk}, as well as the involvement of Ras in this process, we performed immunocytochemical staining of both wild-type PC12 cells and the PC12-M17 subclone treated with these agents. In PC12 and PC12-M17 cells grown under normal conditions (i.e. in high-serum containing medium) p90^{Rsk} immunoreactivity was found in the cytoplasm as well as in the nucleus of both cell lines (data not shown). These observations suggest that a basal level of p90^{Rsk} in the nucleus is not sufficient alone to induce neuronal differentiation in PC12 cells. When immunocytochemical staining was performed in cells cultured in the absence of serum for 2 days, p90^{Rsk} immunoreactivity was detected almost exclusively in the cytoplasm in PC12 as well as in the PC12-M17 subclone (in more than 90% of cells) (Fig. 1 panels a, d). In contrast, when cells were treated with 50 ng/ml NGF for 30 min before fixation, the cytoplasmic localisation of the enzyme was altered in PC12 cells: a strikingly marked translocation of these enzymes from the cytoplasm to the nucleus appeared in approximately 75% of cells (Fig. 1b), however, only a very slight immunostaining was detectable in the nuclei of less than 10% of PC12-M17 cells (Fig. 1e). In the case of ionomycin treatment, p90^{Rsk} displayed patterns of distribution identical to what we observed upon NGF treatment in wild-type PC12 cells (Fig. 1c), however, this nuclear translocation seemed to be unaffected by the blockade of Ras; p90^{Rsk} translocation induced by Ca^{2+} was comparable in PC12-M17 (Fig. 1f) and wild-type PC12 cells.

Phosphorylation of CREB

Since p90^{Rsk} is considered to be the major CREB kinase enzyme in several cell lines, we performed Western blot analysis to examine whether nuclear translocation of p90^{Rsk} is followed by the phosphorylation of CREB also in our experimental system. The phospho-specific anti-CREB antibody used in these experiments recognizes and binds to the phosphorylated form of CREB exclusively, thus providing means to determine the extent of CREB activation. In cells left untreated we were not able to detect phosphorylated CREB protein in either of our subclones (Fig. 2A and B, lane 1). In wild-type PC12 cells CREB phosphorylation occurred in response to both NGF and ionomycin upon 30 min treatment, what is in accordance with the time period necessary for p90^{Rsk} translocation (Fig. 2A, lanes 3 and 5). However, in the PC12-

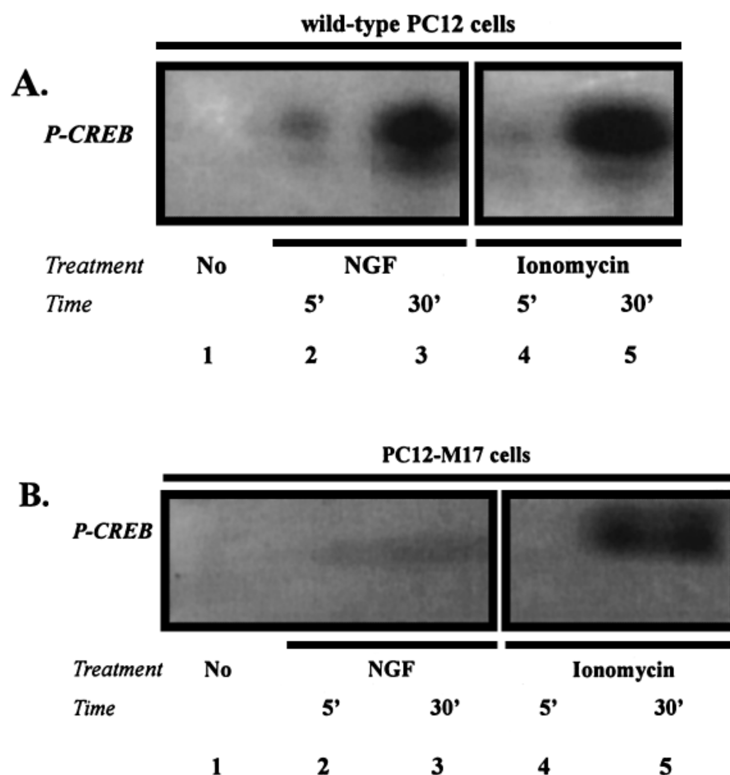

Fig. 2. Detection of CREB phosphorylation in PC12 subclones in response to various treatments. A – Effect of NGF and ionomycin on CREB phosphorylation in wild-type PC12 cells; B – Effect of NGF and ionomycin on CREB phosphorylation in PC12-M17 cells. The agents were used for the indicated time in the following concentrations: NGF (50 ng/ml), ionomycin (0.25 μM)

M17 subclone NGF was unable to induce substantial CREB phosphorylation (Fig. 2B, lanes 2 and 3), confirming the importance of intact Ras in the NGF-stimulated pathway. In contrast, when PC12-M17 cells were treated with ionomycin for 30 min, detectable phosphorylation of CREB occurred in this cell line as well (Fig. 2B, lane 5), although with somewhat reduced intensity than observed in wild-type PC12 cells in response to the same treatment. Our results thus indicate that Ca^{2+}-activation of the $p90^{Rsk}$/CREB axis is highly independent of Ras.

DISCUSSION

Purification and characterization of $p90^{Rsk}$ was first performed in *Xenopus laevis* eggs [1, 11], where it was shown to play a critical role in the regulation of the onset of meiosis II [3, 15]. Human homologs of *Xenopus* $p90^{Rsk}$ were then identified on the

basis of their capacity to phosphorylate the ribosomal protein S6 *in vitro,* and shown to be phosphorylated and activated during initiation of cell proliferation [6] in response to growth factors, phorbol esters and cAMP [7]. Since then, three human isoforms (Rsk1, 2 and 3) have been characterized according to their tissue specific expression [22] and, although their functional specificity remains undefined, a wide variety of substrates of Rsk enzymes have been detected in different cell types, for example, the adhesion molecule L1 [32], the adaptor protein Sos [10], a Na^+-H^+ exchanger enzyme [30], and histone protein H3 [25]. p90^{Rsk} was also reported to take part in cell survival mechanisms by phosphorylating Bad [4, 26] and to be involved in stress signal mediated pathways [20]. Nevertheless, the major role of p90^{Rsk} is believed to be the regulation of gene expression via association with and phosphorylation of transcription factors including c-Fos, Elk-1, SRF, NFkB [2, 5, 12] and especially CREB [34, 35]. Phosphorylation and activation of p90^{Rsk} is thought to be primarily performed by ERKs, moreover, among the substrates of ERKs p90^{Rsk} has proven to be a ubiquitously versatile mediator of ERK signal transduction. Rsk enzymes are coordinately regulated and spatially distributed with ERKs [8, 23] and the two enzymes were found to exist as a heterodimer in their inactive state *in vivo* [17]. However, it should be noted that the kinetics of p90^{Rsk} phosphorylation does not exactly correlate with ERK activation [7, 8], therefore p90^{Rsk} may also be phosphorylated by other protein kinases.

In this paper we tested the possibility of the involvement of p90^{Rsk} in ionomycin-stimulated pathways supporting neuronal differentiation in PC12 cells occurring independently of ERK activation. PC12-M17 cells, expressing a dominant inhibitory Ras protein, do not exhibit neurite outgrowth in response to NGF but are able to undergo neuritogenesis when treated with NGF in combination with the Ca^{2+} ionophore ionomycin [28]. Such a combined treatment is also able to enhance the binding of transcription factors to their promoters and induce gene expression in PC12-M17 cells [24]. However, the neuritogenesis evoked by NGF/ionomycin treatment was shown to be independent of the classic Ras-MEK-ERK pathway (Boglári and Szeberényi, in press), suggesting that protein kinases other than ERKs might take part in the induction of events leading to neurite outgrowth. Different experimental data support the possible involvement of members of the novel protein kinase C family [9], calmodulin dependent kinases [21], as well as the p38 MAPK [16] in this process, however, the role of these enzymes in neuronal differentiation is still controversial. Nevertheless, a strong candidate for the convergence point where NGF and Ca^{2+}-stimulated signals may congregate is the transcription factor CREB. The enzyme shown to be the major CREB kinase is p90^{Rsk}, phosphorylating CREB at its critical regulatory site [35]. Moreover, Rsk2 was also shown to translocate along with ERKs to the nucleus upon depolarization by KCl in PC12 cells [18] and in response to NGF [8, 23]. In this paper we demonstrate that p90^{Rsk} can undergo nuclear translocation under circumstances when ERKs do not: although the combined treatment of PC12-M17 cells with NGF plus ionomycin is not able to induce nuclear translocation of ERKs (Boglári and Szeberényi, in press), p90^{Rsk} enters the nucleus in response to both NGF and ionomycin in wild-type PC12 cells. However,

whereas the NGF-induced translocation is blocked by the presence of dominant inhibitory Ras, ionomycin is able to force Rsk to move to the nucleus in wild-type PC12 as well as in PC12-M17 cells. Moreover, the spatial distribution of $p90^{Rsk}$ is followed by an increase in CREB phosphorylation in both cell lines.

Taken together, our results presented in this paper show that $p90^{Rsk}$ can perform nuclear translocation in PC12 cells stimulated by Ca^{2+} leading to activation of CREB even in the absence of a functioning Ras-ERK signaling pathway. This notion supports the hypothesis that Rsk may participate in conveying the co-stimulatory effect of Ca^{2+} in the process of neuritogenesis. Rsk/CREB activation by ionomycin, however, is by itself not sufficient to trigger neurite formation: it should converge with a currently unidentified, $p140^{Trk}$ NGF receptor originating, Ras independent signaling mechanism to induce process outgrowth [28, Bogl´ari and Szeberényi, in press]. Characterization of this latter pathway would help our understanding of differentiation signaling in PC12 cells.

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