FEBS Letters 474 (2000) 184–188 FEBS 23730

Priming of PC12 cells for semiquantitative microinjection studies involving Ras

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Received 21 March 2000; received in revised form 8 May 2000

Edited by Giulio Superti-Furga

Abstract Nerve growth factor and activated Ras can induce differentiation of rat pheochromocytoma cells (PC12 cells) [Greene and Tischler (1976) Proc. Natl. Acad. Sci. USA 73, 2424–2428] from a chromaffin cell-like morphology into one that resembles sympathetic neurones. We developed a special treatment of PC12 cells which apparently synchronises these cells such that they are more useful for semi-quantitative microinjection studies for signal transduction pathways. This treatment leads to a faster and more reproducible differentiation which faithfully reproduces the involvement of Ras in the process and allows a comparison of the biological activity of different Ras mutants. It shows that G12V and Q61L oncogenic mutants are not equally potent in inducing differentiation. Partial loss-of-function mutations T35S, E37G and Y40C are inactive and even a triple combination of these does not restore full biological activity.

Key words: Ras; Rat pheochromocytoma cells; Oncogene

1. Introduction

Ras genes (H-, N-, K-Ras) are involved in many signal transduction cascades, one of which is the activation of one or more mitogen-activated protein kinase pathways [2–5]. Therefore the development of biological assays to analyse the biological activity of wild-type and mutant Ras genes to understand the function of these signal switch molecules has been a major focus of recent research. Most of these assays are based on the ability of Ras to induce mitogenesis and focus formation in fibroblasts. Genetic evidence from flies and worms has suggested that Ras is apparently equally important for differentiation pathways. Genetic model systems have been the development of photoreceptor cells during the formation of the compound eye of *Drosophila* or the development of a vulva from appropriate precursor cells in *Caenorhabditis elegans* [6]. Ras has also been found to be involved in the signal transduction from the differentiation factor nerve growth factor (NGF) in cultured neurones [7–9].

In the rat pheochromocytoma cell, PC12 [1], it has been shown that transfection of Ras genes or microinjection of Ras proteins into the cell leads to the development of neurites [10–12]. While microinjection of proteins into mammalian cells is a very direct way of assessing their biological potency, its drawback is the usually long development of a cellular response and the lack of reproducibility due to the lack of synchronisation of biological material. Here we have developed a method for priming PC12 cells that allows an easy assessment of biological activity of Ras proteins, and to compare wild-type with different types of mutants.

2. Materials and methods

PC12 cells (clone 251-c/3, a subclone with high NGF responsiveness) were cultivated in 85% Dulbecco's modified Eagle's medium, (1 g/l glucose), 10% horse serum, 5% foetal bovine serum. 5×10^4 cells were seeded per 25 cm^2 Falcon tissue culture flask in 5 ml medium and maintained at 37°C with 10% CO_2. They were passaged every week and split 1:6–1:8. For priming cells were singularised due to trituration with a sterile tip and about 2×10^4 cells per Corning 35 mm tissue culture dish were plated in 2.5 ml culture medium. One hundred ng/ml NGF was added per dish and the cells were incubated for 3 days at 37°C with 10% CO_2. After that the cells were washed three times with medium and further cultivated in 2.5 ml fresh medium without NGF. Directly after retraction of the cells' neurites (usually after 2 days) the cells were microinjected.

Most of the Ras proteins used have been described before, and are expressed and purified using the protocol described earlier [39]. Microinjections were performed on a Zeiss microinjection work station with capillaries with a tip smaller than 0.25 μm in diameter. Proteins were diluted to 150 μM in phosphate-buffered saline or standard protein buffer (50 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$ and 5 mM dithiothreitol) and centrifuged for 2 min before injection. After injection 100 μl penicillin/streptomycin solution was added to the medium. After 24 h cells with neurites were counted. Between 100 and 200 cells were injected per data point and each measurement was repeated at least three (up to six) times. Microinjected cells are scored as those being blown up during the procedure.

3. Results and discussion

Fig. 1 (first columns) shows the results of microinjecting wild-type Ras into PC12 cells. The number of positively scored cells showing neurites (cells bearing neurites longer than two times the cell diameter were scored as positive) varies between 2 and 35% under apparently identical conditions. This is probably due to the cells being in different stages of the cell cycle at the time of microinjection. To obtain more reproducible results and thus be able to use this differentiation assay to assess at least semi-quantitatively the biological activity of Ras and its mutants, we tried to synchronise the cells such that they responded more uniformly to the treatment.

It has been shown before that PC12 cells undergo a change in their potential to grow out neurites in the presence of NGF that has been termed 'priming' [13]. The priming method described involves exposing cells for more than 10 days to NGF and shearing off their neurites. After introducing NGF the

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PII: S0014-5793(00)01602-1

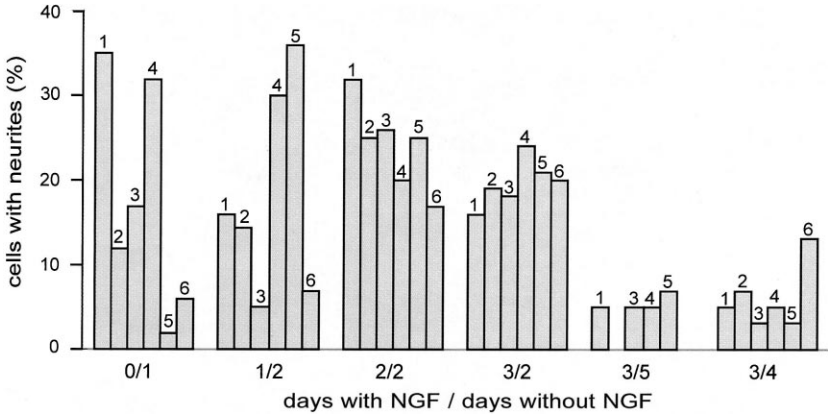

Fig. 1. Priming of PC12 cells for microinjection. PC12 cells were stimulated first with, then without NGF before microinjection of wild-type Ras protein, and are scored as % injected proteins showing neurites with twice the cell body length. The time of induction is indicated below as *x*/*y* where *x* is the number of days the cells have been incubated with, *y* that without NGF. The experiment was repeated at least five times, see parallel rows, to indicate the reproducibility.

cells need just 1–2 days for the development of neurites. Unprimed NGF-treated cells start neurite outgrowth after a lag of about 24 h and require up to 7 days until most cells bear neurites [14]. Primed cells are more responsive to NGF and have been used to detect NGF in goldfish brain [15].

We have tested various preincubation methods which involve pretreatment of cells with and subsequently without NGF for different times as indicated in Fig. 1. Contrary to the Green and Tischler method, we did not mechanically shear off the extensions, but allowed them to retract by themselves. The development of neurites after microinjection of wild-type and mutant Ras highly depends on the culturing conditions. By changing the time periods of stimulation and cultivation with and without NGF it was possible to find the optimal conditions for highly reproducible microinjection experiments. It was also possible to adjust the level of the response of PC12 cells to different levels as indicated (Fig. 1). Since we wanted to compare Ras mutants with higher and lower activity than wild-type, we chose conditions where wild-type Ras induces 20% of the cells to induce neurite outgrowth after 24 h, where normal unprimed cells need more than 48 h for full response, indicating that the lag phase has been shortened. This is particular useful for microinjection since we cannot expect all the Ras mutants to be equally stable under the conditions of the experiment. The major advantage of our priming method as compared to that of Burstein and Greene seems to be that it is more gentle on the cells, as 90% of them survive injection. The method also appears to be much more reproducible and easy to perform. In the following the protocol for microinjection always uses the conditions of treatment of the cells for 3 days with and 2 days without NGF. Because even NGF-untreated PC12 cells sometimes bear small extensions only cells with neurites longer than two times the cell diameter were scored as positive.

3.1. Differentiation is dependent on transcription

One property of cells primed according to Green and Tischler is their ability to grow out neurites in the presence of transcription inhibitors such as actinomycin D, in contrast to unprimed cells. Since our preconditioning involves treatment with NGF for only 3 days we wondered whether neurite induction in these cells requires transcription. Under the conditions reported by Greene and Tischler (8 μM actinomycin D), neurite outgrowth is completely inhibited in the conditioned just as in non-conditioned cells, which means that the process requires RNA synthesis.

Since actinomycin inhibits neurite outgrowth but a 24 h lag phase is not observed under the conditions used in our assay,

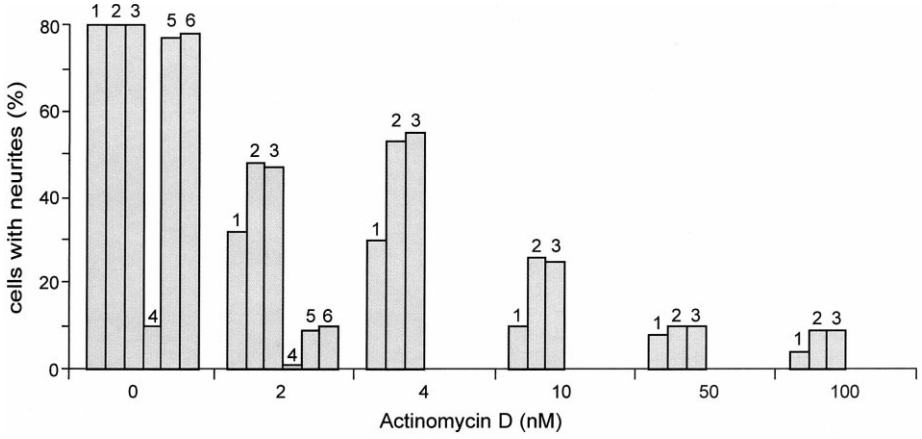

Fig. 2. Effect of varying concentrations of actinomycin D in nM (as indicated) on neurite outgrowth of primed and unprimed PC12 cells. Columns 1–3 are primed, columns 4–6 unprimed cells. Treatment of cells with NGF was for 24 (1,4), 48 (2,5) and 72 (3,6) h. With higher concentrations of actinomycin only primed cells (columns 1–3) were used.

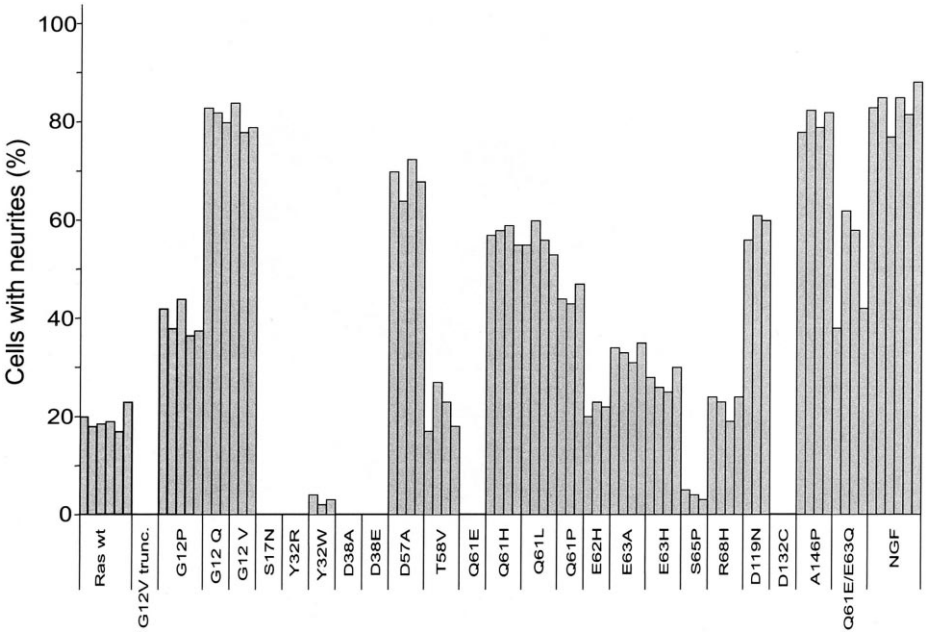

Fig. 3. Biological activity of a panel of wild-type and mutant Ras proteins, as indicated below the columns, on neurite outgrowth of PC12 cells primed by the standard procedure of incubating for 3 days with and 2 days without NGF. The number of columns for each mutant indicates the number of independent experiments.

we assume that a sufficient set of proteins needed for the initiation of the response does exist in the cells after priming, but that some transcription is still necessary for prolonged neurite outgrowth. To test this the inhibition experiment was repeated with lower concentrations of actinomycin D, such that RNA synthesis is only partially blocked (Fig. 2). Even low concentrations of actinomycin D (0.002 μM) inhibit neurite outgrowth, but the effect on primed cells is much weaker than on unprimed cells. In the presence of 4 nM actinomycin D 40–50% of the primed cells are able to grow out neurites during 24 h cultivation with NGF, whereas none of the control cells bear neurites even after 3 days cultivation with NGF.

3.2. Microinjection of Ras proteins

With a suitable assay to measure the biological activity of Ras proteins at hand we tested a panel of mutants with various biochemical properties. Fig. 3 shows that the percentage of neurite producing cells varies between 0 and 85% with different mutants of Ras, and two representative examples are documented in Fig. 4. The variation between different experiments is minimal and the standard error normally ranges from 0 to 4.6%. One can see that oncogenic mutants such as Gly-12 (Fig. 4A) have the strongest biological effect comparable to that of NGF, whereas the Q61 mutants are less efficient in producing a biological readout. As a control, truncated Ras(G12V), residues 1–166, shows no biological activity confirming the necessity of the intact and fully modified C-terminus for the biological activity [16–20]. The Q61E mutants is likely inactive due to the instability of protein [21]. This is corroborated by the Q61E,E63Q double mutant, which preserves the GTPase-negative mutation Q61E, but is stabilised by the E63Q mutation, and is now a potent neurite inducer. It has been shown that mutants with increased dissociation rates for guanine nucleotides are also able to induce transformation or PC12 differentiation. Fig. 3 shows as an example D119N, which is a transforming mutant that has a low affinity for guanine nucleotides [22–25] and has a similar increase in its affinity for xanthine nucleotides [24,25]. Its potency is similar to that of oncogenic Gln61 mutants. The totally conserved Ala-146, whose main-chain nitrogen interacts with the O6 oxygen of the guanine base, is responsible for the specificity of guanine binding. The A146P mutation has very weak affinity for guanine nucleotides (data not shown) and correspondingly is a strong inducer of neurite outgrowth, actually the most biologically potent protein tested by us.

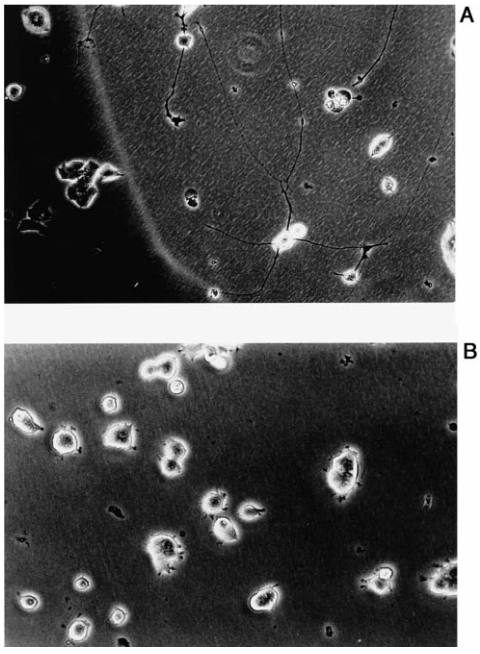

Fig. 4. Representative examples of the microinjection assay, showing PC12 cells 2 days after microinjection of Ras(G12V) (A) and Y32R (B).

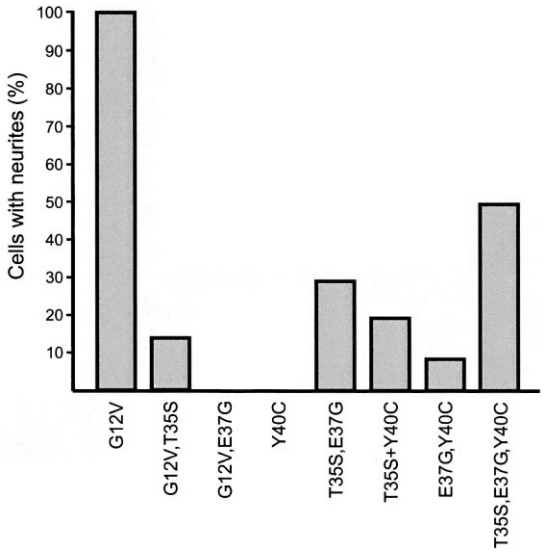

Fig. 5. Effect of single or combinations of partial loss-of-function mutations on neurite outgrowth of primed PC12 cells, relative to the effect of the oncogenic Ras(G12V) mutant, which is 100%.

That the assay is very sensitive to small changes in biological activity is shown by the G12P mutant which was reported to be the only mutation of Gly-12 that is not oncogenic [26], presumably because the intrinsic GTPase is 2–3-fold faster than that of wild-type [27,28]. Since the GTPase activity of p21(G12P) is not stimulated by GAP, it has been proposed that the intrinsic activity is solely responsible for the biological behaviour of p21 mutants [29]. Fig. 3 shows that p21(G12P) is nevertheless twice as active as wild-type p21 and that the increased GTPase rate of the mutant cannot totally compensate for the loss of GAP stimulation. Other mutants with an effect intermediate between wild-type and oncogenic Ras are the loop L4 mutants such as E62H, E63A, E63A, most likely due to the reduced interaction with GAP as shown by the three-dimensional structure of the complex [30]. Y32R (Fig. 4B), although only partially reactive in the GAP-mediated GTPase reaction, is nevertheless not biologically active due to its inability to react with effectors (C. Herrmann, unpublished).

Recently it has become clear that Ras uses more than one pathway to mediate the downstream response such as transformation. Mutants of Ras in the effector region have been identified which are supposed to be specifically blocked in a particular pathway [31–38]. Such partial loss-of-function mutants can be used to delineate the specific downstream signalling components of a particular pathway. It is generally believed that E37G, T35S and Y40C are specific inducers of the RalGEF, Raf and PI-3 kinase pathways, respectively. These mutants, in the context of the GTPase-negative mutant G12V, were thus tested in the context of the PC12 differentiation assay presented here. Fig. 5 shows that the single mutants have drastically decreased differentiation activity, which according to common wisdom would mean that all three Ras pathways mentioned are involved in the differentiating activity of the protein. It has been postulated that combining partial losses-of-function restores biological activity. However, as shown in Fig. 5, none of the double combinations of single point mutations restores a large amount of biological activity, and only the triple combination restores 50% activity. This confirms our detailed biochemical analysis of Ras–effector interactions which show that all the commonly used partial loss-of-function mutations have reduced affinities to different effectors (data not shown), which would make it unlikely that the combinations of the mutants would fully restore the pathways and thus biological function. As a caveat it should be noted that the results obtained with the PC12 cell line cannot necessarily be applied to a more physiological system such as postmitotic neurones although Ras has been shown to be responsible for survival and maintenance of differentiation of primary neurones as well [7,8].

Acknowledgements: We thank Doro Vogt for cloning and preparation of Ras mutants, Brigitte Oeke for help in microinjection studies and Rita Schebaum for secretarial assistance.

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