The *ras* oncogene inhibits growth factor inducibility of early response genes, and promotes selectively expression of NGFI-A in a PC12 cell line

José Miguel Cosgaya, Ana Aranda*

Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, Arturo Duperier 4, 28029 Madrid, Spain

Received 8 January 1999

Abstract Expression of oncogenic Ras in UR61 cells (a PC12 subclone) results in neuronal differentiation. We have observed that the oncoprotein selectively increased the levels of NGFI-A transcripts, but was unable to induce NGFI-B or c-*fos* transcripts. In contrast, nerve growth factor (NGF) elicited a strong induction of the three immediate early genes (IEGs). Thus, activation of Ras alone is sufficient for the induction of NGFI-A by NGF, whereas an additional pathway(s), besides Ras, is required for the stimulation of NGFI-B and c-*fos* gene expression. These results show that the acquisition of a neuronal phenotype does not correlate with induction of IEG expression. Additionally, Ras markedly reduces the response of the three genes to NGF and to other growth factors. This attenuation could reflect a negative regulatory mechanism acting on signalling pathways normally stimulated by growth factor receptors.

Key words: Ras; Nerve growth factor; Immediate early gene; Retinoic acid; PC12 cell

1. Introduction

Upon incubation with neurotrophic factors, such as nerve growth factor (NGF) [1] and fibroblast growth factor (FGF) [2], or after expression of the Ras oncogene [3,4], PC12 cells acquire a phenotype resembling sympathetic neurons. The neuron-like differentiation of PC12 cells is induced by NGF through a sustained stimulation of a signalling pathway, the Ras-MAPK (mitogen-activated protein kinase) cascade [5]. This results in the rapid and transient induction of a set of genes called immediate early genes (IEGs) which encode several protooncogenes and transcription factors [6,7]. Epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), which also bind to tyrosine kinase receptors, stimulate proliferation and cause a transient MAPK activation [8], without causing differentiation of PC12 cells. However, both neurotrophic and mitogenic factors induce expression of IEGs in these cells.

Retinoic acid, a ligand of nuclear receptors which belong to the steroid/thyroid hormone nuclear receptor superfamily, has also pronounced effects on PC12 cell proliferation, differentiation and gene expression [9–11]. We have recently shown that RA attenuates the response of NGFI-B and c-Fos to NGF, but does not alter NGF induction of NGFI-A [12]. NGFI-A (also called *zif*268, *egr*-1, *krox*24, TIS8 and D2) codes for a member of the zinc-finger transcriptional activator family [13], and NGFI-B (also called N10 and *nur*77) codes for an orphan receptor member of the steroid/thyroid hormone receptor superfamily [14].

*Corresponding author. Fax: +34 (91) 5854587.
E-mail: aaranda@iib.uam.es

In this study we have analyzed the effect of Ras on the expression of c-*fos*, NGFI-A, and NGFI-B in UR61 cells, a subclone of PC12 cells which contains a corticosteroid-inducible N-*ras* oncogene. NGF does not induce neurite outgrowth in UR61 cells, but incubation with dexamethasone causes expression of Ras and extensive neurite extension [15]. Our data show that expression of oncogenic Ras in UR61 cells induces NGFI-A, but not c-*fos* or NGFI-B transcripts. In addition, the three IEGs are strongly induced by NGF and other ligands of tyrosine kinase receptors, and the oncoprotein markedly blocks these responses. These results show that in these cells the acquisition of a neuronal phenotype does not correlate with induction of IEG expression.

2. Materials and methods

2.1. Cell cultures

UR61 cells were cultured as previously described [10,11] in RPMI medium containing 10% donor horse serum (Quality Biological Inc.) and 5% fetal calf serum (GIBCO). UR61 cells were derived from PC12 cells following stable transfection with a plasmid containing the transforming mouse N-*ras*^{Val12} oncogene under control of the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter [15].

2.2. RNA extraction and hybridization

Total RNA was extracted from the cell cultures with guanidine thiocyanate. The RNA was run in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes (Nytran) for Northern blot analysis. The RNA was stained with 0.02% methylene blue, and the blots were sequentially hybridized with cDNA probes for NGFI-A [13], NGFI-B [14] and c-*fos* [16] labeled by random oligonucleotide priming. Hybridizations were carried out at 42°C with 50% formamide and the most stringent wash was at 42°C with 0.1×SSC-0.1% SDS. Quantification of mRNA levels was carried out by densitometric scan of the autoradiograms. The values obtained were always corrected for the amount of RNA applied in each lane which was determined by densitometry of the methylene blue stained membranes.

3. Results

The left panel in Fig. 1 shows that, confirming our previous observations [17], NGFI-A mRNA levels were significantly induced in UR61 cells treated with 100 nM dexamethasone for 8 h. Since dexamethasone treatment did not induce NGFI-A mRNA levels in parental PC12 cells [12], this result indicates that activated Ras is responsible for stimulation of NGFI-A gene expression by the steroid in UR61 cells. As illustrated in the right panel, NGFI-A transcripts were detectably increased at 3 h of dexamethasone treatment, were maximal between 8 and 24 h, and returned to basal levels after 36–48 h. In contrast to the induction of NGFI-A, NGFI-B or c-*fos* mRNA remained undetectable upon incubation of UR61 cells with dexamethasone for time periods ranging from 30 min

PII: S0014-5793(99)00101-5

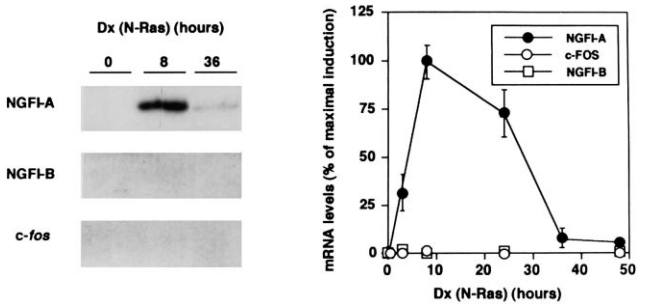

Fig. 1. Expression of N-Ras induces NGFI-A, but not NGFI-B or c-*fos* expression in UR61 cells. These cells contain the N-*ras*^{Val12} oncogene under control of the glucocorticoid-inducible MMTV promoter. The left panel shows representative Northern blots of 30 μg of total RNA obtained from cells incubated with 100 nM dexamethasone (Dx) for 0, 8 and 36 h. The right panel shows the quantification of IEG mRNA levels in cells treated from various intervals (0, 0.5, 3, 8, 24, 36 and 48 h) with Dx. Data are mean ± S.D. values and are expressed as percentages of the maximal mRNA values obtained.

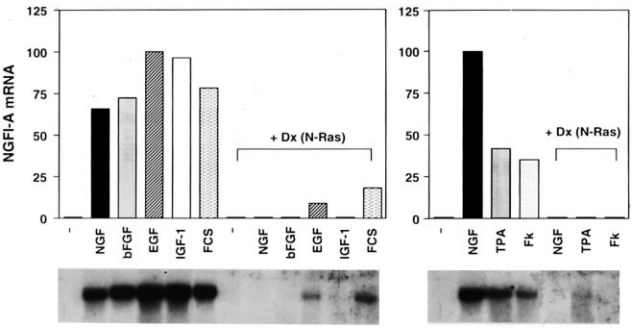

Fig. 3. Influence of Ras on IEG response to different stimuli. UR61 cells were incubated for 36 h in the presence or absence of 100 nM dexamethasone (Dx), and for the last 30 min with: 50 ng/ml NGF, 17 ng/ml bFGF, 60 ng/ml EGF, 38 ng/ml IGF-1 or 20% fetal calf serum (FCS) (left panel); or with 50 ng/ml NGF, 100 nM TPA or 10 μM forskolin (Fk) (right panel). Northern blot analysis was carried out with 30 μg total RNA and a labeled NGFI-A cDNA probe (lower panels). The upper panels show the quantification of the NGFI-A transcripts shown in the lower panels. The data are expressed as the percent of the maximal mRNA value obtained which was considered as 100%.

to 48 h. These results demonstrate a differential response of different IEGs after expression of Ras.

IEG response to NGF was also examined in UR61 cells. Fig. 2 shows the levels of NGFI-A, NGFI-B and c-*fos* transcripts. Basal levels were undetectable in the absence of NGF, but were strongly increased 30 min after NGF treatment. Thereafter, the three mRNAs decreased rapidly, being barely detectable at 2 h. Transcription of these genes does not require 'de novo' protein synthesis, and inhibitors of protein synthesis prolong transcription and stabilize the mRNAs [18]. Fig. 2 illustrates that NGF stimulation of UR61 cells in the presence of cycloheximide leads to superinduction of IEG transcripts, which under these conditions reach the highest levels at 2 h. Therefore, although c-*fos* and NGFI-B mRNAs were not induced after Ras expression, these mRNAs showed a normal response to NGF in UR61 cells. To analyze the effect of activated Ras on the inducibility of IEGs by NGF, the response to NGF was examined in UR61 cells

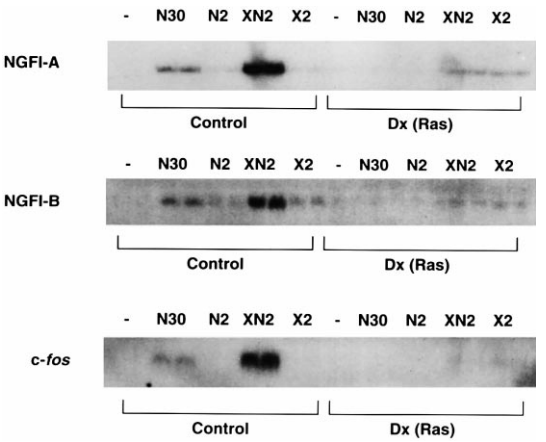

Fig. 2. Ras abolishes the IEG response to NGF. UR61 cells were pretreated with medium alone (Control) or with 100 nM dexamethasone (Dx), which induces expression of the N-*ras*^{Val12} oncogene, for 36 h. The cells were treated for the last 30 min or 2 h with 50 ng/ml NGF (N) and/or 5 μg/ml cycloheximide (X) as indicated. N30, NGF 30 min; N2, NGF 2 h; XN2, cycloheximide+NGF 2 h; X2, cycloheximide 2 h. Northern blot analysis was performed with 30 μg total RNA and labeled NGFI-A, NGFI-B and c-*fos* cDNA probes. Representative blots obtained from duplicate cultures for each treatment are shown.

treated with dexamethasone for 36 h. This treatment essentially abolished the response to NGF. Expression of Ras was even able to block the maximally induced levels of NGFI-A, NGFI-B and c-*fos* transcripts obtained in UR61 cells incubated with the combination of NGF plus cycloheximide. Dexamethasone did not reduce NGF inducibility in parental PC12 cells [12], showing that the repressive effect of the corticosteroid is due to the expression of oncogenic Ras.

We next tested whether IEG activation by other factors would be also repressed by oncogenic Ras in UR61 cells. Fig. 3 shows that treatment with bFGF, EGF or IGF-1 increased NGFI-A transcripts to similar levels as did NGF or serum. In addition, inducibility by the different factors was significantly reduced after expression of the *ras* oncogene (left panel). This attenuation is not restricted to growth factors since, as shown in the right panel, the response to TPA or forskolin was also blocked in UR61 cells expressing Ras. Identical results were obtained for NGFI-B and c-*fos*, since their mRNAs were induced by the different stimuli, but were undetectable in UR61 cells treated with dexamethasone (data not shown).

We have previously shown that in PC12 cells RA attenuates the response of the NGFI-B and c-*fos* genes to NGF, whereas it does not alter NGFI-A induction [12]. Therefore, it was of interest to analyze whether RA could also modulate IEG response to Ras. For this purpose, the mRNA levels of NGFI-A, NGFI-B, and c-*fos* were determined in untreated control UR61 cells and in cells incubated for 36 h with dexamethasone, RA or both. For each treatment, cells were incubated with medium alone, NGF and/or cycloheximide. As illustrated in Fig. 4, treatment with RA decreased NGFI-B and c-*fos* induction by NGF, as well as the superinduction caused by NGF plus cycloheximide in UR61 cells. These results are similar to those found in the parental PC12 cells. In addition, expression of Ras blocks the response to NGF, and both in the absence and the presence of RA, NGFI-B and c-*fos* transcripts were basically undetectable in the cells treated with dexamethasone. Again the response of the NGFI-A gene was different from that of NGFI-B or c-*fos* genes. As illustrated in Fig. 5, treatment with RA did not alter basal NGFI-

A mRNA levels or the response to NGF and cycloheximide. Again, expression of Ras significantly blocked the induction of NGFI-A by NGF. This repressive effect was not affected by RA, as shown by the low levels of NGFI-A transcripts observed in cells pretreated with the combination of RA and dexamethasone and stimulated with NGF. Unexpectedly, incubation with cycloheximide caused a strong induction of NGFI-A mRNA levels in cells pretreated with RA and dexamethasone. These levels were close to the maximal superinduced levels in control cells, and were higher than those found in cells incubated with NGF for 30 min under control conditions. This induction by cycloheximide was not found after an independent treatment with dexamethasone or RA, and the combination of both agents was required for the stimulation of NGFI-A transcripts.

4. Discussion

Expression of oncogenic Ras, which causes neuronal differentiation of UR61 cells [15] results in a strong induction of NGFI-A transcripts. In contrast, expression of Ras was unable to induce NGFI-B and c-*fos* transcripts in these cells. Although the NGFI-A, NGFI-B and c-*fos* genes bear similar expression kinetics following NGF stimulation, and despite the extensive similarity among the promoter elements involved, their induction must have different components since their response to Ras is different. The lack of c-*fos* induction by Ras in UR61 cells [19] is consistent with the finding that no significant elevation of c-*fos* transcripts has been found in fibroblasts transformed by oncogenic Ras [20]. In contrast with these observations, it has been described that c-*fos* transcripts are induced in PC12 cells infected with retrovirus vectors carrying an activated Ha-*ras* oncogene [21]. This discrepancy might be due to the fact that UR61 cells express N-*ras* rather than Ha-*ras*. Although N-, Ha- and K-*ras* exhibit a very high degree of homology, several recent reports support specific biological roles for the Ras isoforms [22,23].

Our results suggest that activation of the Ras pathway alone is sufficient for stimulation of NGFI-A gene expression in UR61 cells, whereas additional pathway(s), besides Ras, appear to be required for stimulation of NGFI-B and c-*fos* gene expression. Recent data confirm the participation of more than one signalling pathway in c-*fos* gene induction by the neurotrophins. It has been described that both the serum response element (SRE) [24], and the cyclic AMP response element (CRE) of the c-*fos* promoter are critical for NGF activation of c-*fos* gene transcription [25]. A NGF-inducible, Ras-dependent protein kinase, which was identified as RSK2, and catalyzes the phosphorylation of CREB (the cyclic AMP response element-binding protein), was found to trigger this activation [25,26]. In addition, a very recent report [27] demonstrates that the stimulation of neuronal c-*fos* expression by neurotrophins involves at least two signalling pathways: the Ras-dependent pathway and a calcium/calmodulin-dependent kinase pathway.

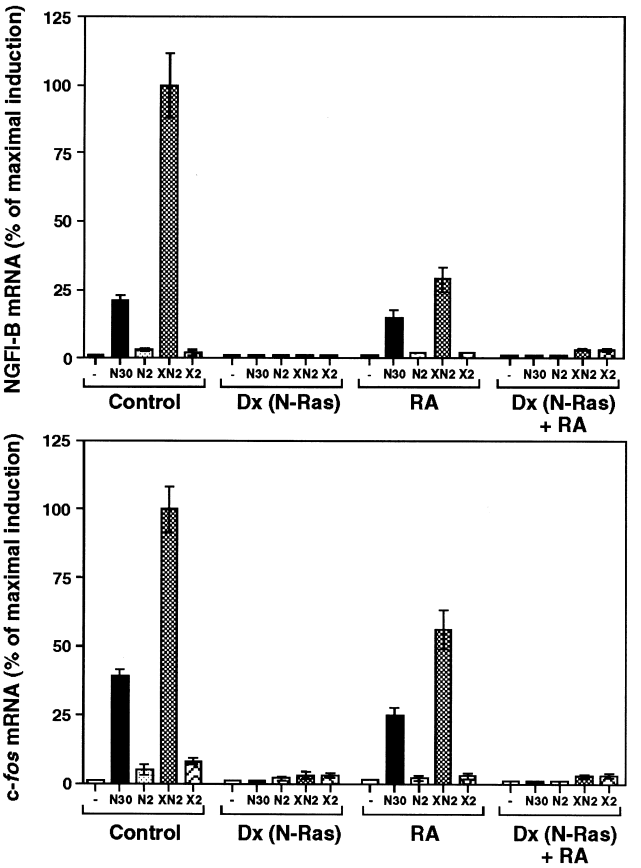

Fig. 4. Combined effects of NGF, retinoic acid (RA) and Ras on IEG expression in UR61 cells. The cells were treated with medium alone (Control), 1 μM RA or 100 nM dexamethasone (Dx) for 48 h. For the last 30 min or 2 h the cells were incubated with 50 ng/ml NGF (N) and/or 5 μg/ml cycloheximide (X) as indicated. -, control; N30, NGF 30 min; N2, NGF 2 h; XN2, cycloheximide+NGF 2 h; X2, cycloheximide 2 h. Northern blot analysis was carried out with 30 μg total RNA and labeled NGFI-B (upper panel) and c-*fos* (bottom panel) cDNA probes. The blots were quantitated by densitometry, and the values obtained were corrected for the amount of RNA applied to each lane. The data for each mRNA are expressed as the percent of the maximal superinduced levels (obtained in cells incubated with NGF and cycloheximide), which were arbitrarily set to 100%. The data represent the mean values ± standard deviation obtained from two independent experiments performed in duplicate.

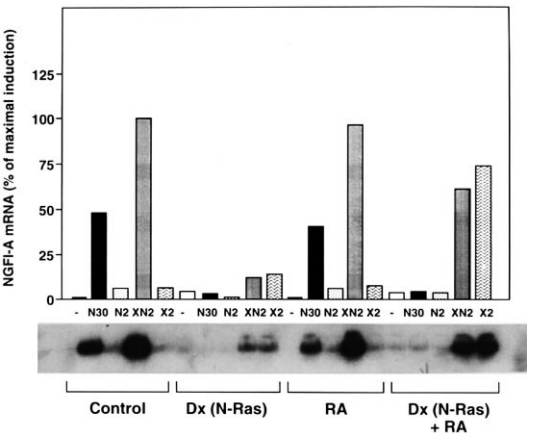

Fig. 5. RA modulates the influence of Ras on NGFI-A expression. UR61 cells were first incubated for 36 h under control conditions or in the presence of 100 nM dexamethasone (Dx), 1 μM RA, or the combination of both. The cells were then treated as in Fig. 4 with NGF (N) or cycloheximide (X). N30, NGF 30 min; N2, NGF 2 h; XN2, cycloheximide+NGF 2 h; X2, cycloheximide 2 h. Northern blot analysis was performed with total RNA and a labeled NGFI-A cDNA probe. The Northern blot shown in the lower panel was quantitated and the data are expressed in the upper panel as the percent of the maximal mRNA levels obtained in cells incubated with NGF and cycloheximide. Similar results were obtained in an independent experiment performed with duplicate cultures for each treatment.

The participation of more than one pathway in the induction of NGFI-B and c-*fos* by NGF would be also compatible with the effect of RA on this response. Since RA does not alter NGFI-A induction by NGF, activation of the Ras pathway would not be repressed by RA. In contrast, treatment with RA would reduce the activity of the Ras-independent pathway stimulated by NGF, allowing a partial induction through Ras, and resulting in a decreased response of the NGFI-B and c-*fos* genes to the neurotrophin.

A previous study has shown that expression of Ras in UR61 cells results in inhibition of NGF-induced c-*fos* transcriptional activation [19]. Our results demonstrate that attenuation of the NGF response by Ras is not restricted to c-*fos* but is a more general property of other IEGs, since the response of NGFI-B and NGFI-A to NGF is also blocked. Furthermore, this attenuation is extensive to the action of other growth factors. Among the growth factors ligands of tyrosine kinase receptors tested, NGF and bFGF induce PC12 cell differentiation, whereas IGF-1 and EGF cause cell growth. However, all of them induced transcripts of the three IEGs with a similar potency, and their action was blocked by Ras in UR61 cells. Because the Ras protein is involved in the signalling pathway of tyrosine kinase receptors, this attenuation could reflect a negative regulatory mechanism acting on signalling pathways normally stimulated by growth factor receptors. Since the induction of the three IEGs by serum and compounds that stimulate protein kinase C and protein kinase A was also repressed by Ras, these stimuli and the growth factors likely share at least some of the pathways involved in these processes. In this respect, all growth factors and compounds analyzed can activate the MAPK/ERK pathway, and it is known that some proteins that are implicated in the Ras pathway, such as Raf or the guanine nucleotide exchanger Sos, can be phosphorylated and inactivated by MAPK [28,29]. Also, as a possible step in the desensitization mechanism, some tyrosine kinase receptors can bind and become phosphorylated by MAPK [30].

It has been reported that RA can increase the expression of NGFI-A in some cell types but not in others [31,32]. The lack of response has been attributed to the presence of nuclear proteins responsible for cell-type specific suppression, which might be involved in the unresponsiveness of the NGFI-A gene to RA in PC12 cells. This is supported by the finding that, in UR61 cells, RA is able to induce NGFI-A mRNA levels in the absence of protein synthesis, a condition in which putative suppressor proteins with a short half-life could be removed. In any event, this removal would not be sufficient by itself, because the effect of RA was only observed after expression of Ras. The modulation of the IEG response shows the existence of a complex cross-talk between the Ras and RA signalling pathways which could have important consequences in PC12 cell proliferation, differentiation or function. Independently of the mechanism(s) by which RA cooperates with Ras to induce NGFI-A expression when protein synthesis is blocked, this effect is not a general property for all IEGs, since under the same conditions stimulation of NGFI-B or c-*fos* was not observed. This finding again demonstrates the divergent regulation of NGFI-A as compared to the two other IEGs.

Induction of IEG expression is assumed to play a key role in proliferation and neuronal differentiation. However, our data show that NGF elicits a IEG response in the morphologically unresponsive UR61 cells which is identical to that observed in the parental PC12 cells where the neurotrophin promotes neurite outgrowth. Moreover, in UR61 cells expression of oncogenic Ras, which causes extensive neuronal differentiation, does not produce transcription of the c-*fos* and NGFI-B genes and blocks induction by growth factors. These results show that IEG expression does not correlate directly with the development of a neuronal phenotype in these cells.

Acknowledgements: We thank Angel Pellicer for the UR61 cells. This work has been supported by grants PB94-0094 and PB97-0135 from the DGES.

References

[1] Greene, L.A. and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2424–2428.
[2] Togari, A., Baker, D., Dickens, G. and Guroff, G. (1983) Biochem. Biophys. Res. Commun. 114, 1189–1193.
[3] Bar-Sagi, D. and Feramisco, J.R. (1985) Cell 42, 841–848.
[4] Noda, M., Ko, M., Ogura, A., Liu, D.G., Amano, T., Takano, T. and Ikawa, Y. (1985) Nature 318, 73–75.
[5] Segal, R.A. and Greenberg, M.E. (1996) Annu. Rev. Neurosci. 19, 463–489.
[6] Herschman, H.R. (1991) Annu. Rev. Biochem. 60, 281–319.
[7] Miranti, C.K., Ginty, D.D., Huang, G., Chatila, T. and Greenberg, M.E. (1995) Mol. Cell. Biol. 15, 3672–3684.
[8] Marshall, C.J. (1995) Cell 80, 179–185.
[9] Cosgaya, J.M., Garcia-Villalba, P., Perona, R. and Aranda, A. (1996) J. Neurochem. 66, 89–98.
[10] Cosgaya, J.M., Perona, R. and Aranda, A. (1997) Oncogene 14, 579–587.
[11] Cosgaya, J.M., Recio, J.A. and Aranda, A. (1997) Oncogene 14, 1687–1696.
[12] Cosgaya, J.M., Perez-Juste, G. and Aranda, A. (1998) FEBS Lett. 429, 254–258.
[13] Ryseck, R.P., Macdonald-Bravo, H., Mattei, M.G., Ruppert, S. and Bravo, R. (1989) EMBO J. 8, 3327–3335.
[14] Milbrandt, J. (1988) Neuron 1, 183–188.
[15] Guerrero, I., Pellicer, A. and Burstein, D.E. (1988) Biochem. Biophys. Res. Commun. 150, 1185–1192.
[16] Curran, T. and Verma, I.M. (1984) Virology 135, 218–228.
[17] Cosgaya, J.M. and Aranda, A. (1996) Oncogene 12, 2651–2660.
[18] Edwards, D.R. and Mahadevan, L.C. (1992) EMBO J. 11, 2415–2424.
[19] Thomson, T.M., Green, S.H., Trotta, R.J., Burstein, D.E. and Pellicer, A. (1990) Mol. Cell. Biol. 10, 1556–1563.
[20] Yu, C.L., Prochownik, E.V., Imperiale, M.J. and Jove, R. (1993) Mol. Cell. Biol. 13, 2011–2019.
[21] Sassone-Corsi, P., Der, C.J. and Verma, I.M. (1989) Mol. Cell. Biol. 9, 3174–3183.
[22] Hamilton, M. and Wolfman, A. (1998) Oncogene 16, 1417–1428.
[23] Maher, J., Baker, D.A., Manning, M., Dibb, N.J. and Roberts, I.A. (1995) Oncogene 11, 1639–1647.
[24] Visvader, J., Sassone-Corsi, P. and Verma, I.M. (1988) Proc. Natl. Acad. Sci. USA 85, 9474–9478.
[25] Ginty, D.D., Bonni, A. and Greenberg, M.E. (1994) Cell 77, 713–725.
[26] Xing, J., Ginty, D.D. and Greenberg, M.E. (1996) Science 273, 959–963.
[27] Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M. and Greenberg, M.E. (1997) Neuron 19, 1031–1047.
[28] Cherniack, A.D., Klarlund, J.K. and Czech, M.P. (1994) J. Biol. Chem. 269, 4717–4720.
[29] Ueki, K., Matsuda, S., Tobe, K., Gotoh, Y., Tamemoto, H., Yachi, M., Akanuma, Y., Yazaki, Y., Nishida, E. and Kadowaki, T. (1994) J. Biol. Chem. 269, 15756–15761.
[30] Williams, R., Sanghera, J., Wu, F., Carbonaro-Hall, D., Campbell, D.L., Warburton, D., Pelech, S. and Hall, F. (1993) J. Biol. Chem. 268, 18213–18217.
[31] Edwards, S.A., Darland, T., Sosnowski, R., Samuels, M. and Adamson, E.D. (1991) Dev. Biol. 148, 165–173.
[32] Suva, L.J., Towler, D.A., Harada, S., Gaub, M.P. and Rodan, G.A. (1994) Mol. Endocrinol. 8, 1507–1520.