

Opposite effects of the Hsp90 inhibitor Geldanamycin: induction of apoptosis in PC12, and differentiation in N2A cells

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Abstract The inhibitor of the Hsp90 chaperone Geldanamycin has been reported to have several cellular effects, such as inhibition of v-src activity or destabilization of Raf-1 among others. We show now that Geldanamycin treatment induces different phenotypes in different cell lines. In PC12 cells, it triggers apoptosis, whereas in the murine neuroblastoma N2A, it induces differentiation with neurite outgrowth. Geldanamycin effects cannot be mimicked by inhibition of the c-src protein tyrosine kinases, and nerve growth factor does not protect PC12 cells from apoptosis. Mitogen-activated protein kinase activities ERK and JNK are activated differently according to cell type: in PC12 cells JNK is activated, and its inhibition abolishes apoptosis, but not ERK; in N2A cells, both ERK and JNK are activated, but with peak activities at different times. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Geldanamycin; Raf-1; Mitogen-activated protein kinase; Apoptosis; Differentiation

1. Introduction

Cells in pluricellular organisms are always challenged by different signals from the external milieu that direct them to one of four possible outcomes: proliferation, differentiation, apoptosis or senescence. The specific signals or inputs towards these pathways are not always well understood, and almost never are exclusive for any given pathway. Growth factors induce proliferation through interaction with tyrosine kinase receptors and a complex recruitment of protein kinases that ends up in the induction of gene expression [1,2]. The same kind of signaling process often also induces differentiation [3].

One interesting property of several signaling pathways is the association of some of their components with different proteins that help to maintain a stable spatial organization, like scaffolding proteins [4] or chaperones that help to maintain an activatable state. Of these chaperones, the multicomponent Hsp90 system [5] is found complexed with steroid hormone receptors [6,7], cyclin-dependent kinases [8], src

[9,10], Raf-1 kinase [11,12] or the kinase suppressor of ras [13]. At least for Raf-1 kinase, it has also shown that p50^{cdc37} is an essential component of this protein complex [14]. It seems, therefore, that Hsp90 has an essential function in signal transduction in addition to its role in the response to stress [15–17].

The function of Hsp90 is inhibited by Geldanamycin (GA), a benzoquinone ansamycin antibiotic previously proposed as an antitumor drug because its inhibition of v-src and p185^{c-erbB-2} tyrosine kinases. Schulte and co-workers have shown that GA treatment induces destabilization and degradation of Raf-1 kinase mediated by the proteasome [18–21].

The present study shows that GA treatment affects in different ways various cell lines, and that these effects can be related in part to the ability of GA to induce degradation of Raf-1.

2. Materials and methods

2.1. Reagents and plasmids

Gö6976, SB203580 and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) were from Calbiochem. Myelin basic protein (MBP) was from Sigma. GA was a kind gift of the Drug Synthesis and Chemistry Branch, Development Therapeutics Program, division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). Antibodies were from Santa Cruz (Santa Cruz, CA, USA), except monoclonal antibody against c-src, a gift of Dr. J. Martín-Pérez (from this institute). The GST-c-jun(1-79) construct was supplied by Dr. M. Karin, University of California, San Diego, CA, USA. Nerve growth factor (NGF) (Sigma) was supplied by Dr. A. Aranda, from this institute.

2.2. Cell culture

The neuroblastoma cell line N2A, obtained from the European Culture Collection (ECACC #98121404), was provided by Dr. A. Pérez-Castillo; PC12 cells were provided by Dr. A. Aranda, both from our Institute. N2A cells were grown at 37°C in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine and 40 mg/ml gentamicin; PC12 cells were grown with 5% fetal calf serum and 10% donor horse serum in the same medium. Cells were subcultured twice weekly and were renewed every 10 passages. In all cases, cells were plated 24 h prior to the experiments.

2.3. Measurement of cell death

The extent of apoptosis was quantified with the Cell Death Detection ELISA kit from Roche (this kit estimates the amount of cytoplasmic, small molecular weight, histone-associated DNA), or by flow cytometry [22]; cells were analyzed in a Becton-Dickinson FACScan. Data are expressed as percentage of propidium iodide-positive cells with hypodiploid content of DNA.

2.4. Assay of extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) activities

After appropriate treatments (see individual experiments), approximately 8×10^5 cells (in 60 mm diameter plates) were washed twice

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Abbreviations: ERK, extracellular signal-regulated kinase; GA, Geldanamycin; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NGF, nerve growth factor; PI3K, phosphatidylinositol-3'-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine

with PBS and lysed with 300 μ l of lysis buffer (20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β -glycerophosphate, 2.5 mM $MgCl_2$, 2 mM *o*-vanadate, 1 mM DTT, 1% NP-40 and protease inhibitor mix (10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 mM benzamide and 1 mM pMSF). Cells were scraped off the plate and incubated at 4°C for 20 min. The cell lysate was centrifuged 15 min at 20 000 $\times g$ at 4°C and the supernatant stored at -70°C until use. Protein (75–200 μ g) was immunoprecipitated with 5 μ l of polyclonal anti-ERK 2 or anti-JNK1 antibodies and incubated 1 h at 4°C. Immunocomplexes were isolated by incubation with 20 μ l of 50% protein A-Sepharose (Sigma) in lysis buffer for 1 h at 4°C with gentle end-over-end rotation, and centrifuged for 1 min. Supernatant was discarded and the beads washed three times (0.5 ml each) with PBS, 2 mM *o*-vanadate, 1% NP-40, once with 0.1 M Tris-HCl, pH 7.5, 0.5 M LiCl and once with kinase buffer (20 mM HEPES, pH 7.5, 2 mM DTT, 20 mM β -glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 20 mM $MgCl_2$, 0.1 mM *o*-vanadate) without ATP. After the last wash, the beads were incubated at 30°C for 20 min in 30 μ l kinase buffer plus 20 μ M ATP,

1–2 μ Ci [γ - 32 P]ATP (4500 Ci/mmol, ICN, Costa Mesa, CA, USA) and 5 μ g MBP for ERK2 or GST-c-jun(1-79) for JNK1. The reaction was terminated with five times concentrated sample buffer, boiled 3 min and electrophoresed on 15% (or 10% for JNK1) SDS-polyacrylamide gels. Gels were briefly stained and fixed, dried and exposed to X-ray film (Amersham hyperfilm, Amersham, UK). Activity was quantified in a Packard instant imager. Previous experiments performed with antibodies against both isoforms of ERK showed that ERK1 represents less than 10% of the total ERK activity in these cells, having the same activation profiles after stimulation with phorbol esters. Similarly, pull down experiments of JNK activity using the GST-c-jun fusion protein bound to glutathione-Sepharose beads, showed no difference between total and JNK1 profiles. Therefore, only ERK2 and JNK1 were studied in this work.

2.5. Other methods

Microphotographs were taken with Nomarski optics in a Zeiss Axiophot microscope. Protein was determined with the BCA protein

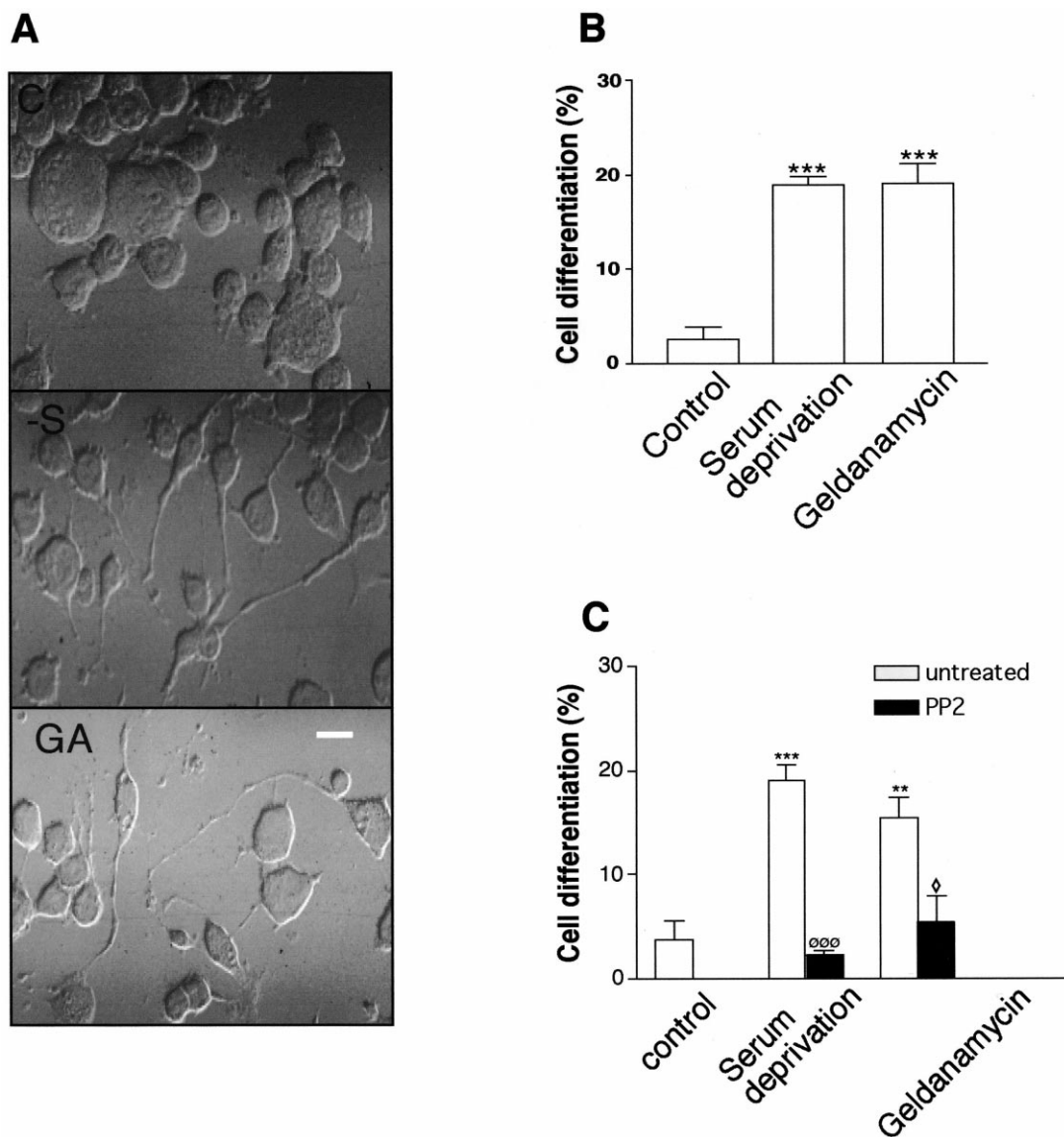


Fig. 1. Effect of GA on N2A cells. A: Morphological differentiation (C, control cells with no treatment; -S, cells deprived of serum for 24 h; GA, cells grown for 24 h in the presence of 2 μ M GA); white bar represents 20 μ m. B: Quantification of N2A cell differentiation after serum withdrawal or GA treatment; cells were scored as positive when they had processes larger than two cell diameters. Data are expressed as mean $\pm$ standard error of the percentage of cells bearing processes in four randomly chosen photographic fields. C: Effect of the c-src inhibitor PP2 on N2A cell differentiation; cells were treated with GA or deprived of serum as described above in the presence or absence of 5 μ M PP2. Quantification of differentiated cells was as above. *** P < 0.001 with respect to control cells; ** P < 0.01 with respect to control cells; $\diamond$ P < 0.05 with respect to cells treated with GA.

assay reagent (Pierce, Rockford, IL, USA). Gel electrophoresis and immunoblotting was done according to conventional protocols; antigen detection was done by Enhanced Chemiluminescence (ECL, Amersham). Statistical analysis, one-way non-paired ANOVA with Turkey's post test comparison, was done with the Prism v.2.0a program (GraffPad Software).

3. Results

3.1. Effect of GA on N2A cells

Treatment of N2A cells with GA resulted in differentiation, shown by neurite extension (Fig. 1A). This effect is equivalent to serum deprivation, as shown in Fig. 1A, and in Fig. 1B in a more quantitative way. This effect cannot be attributed to c-src inhibition, as a specific inhibitor of src activity, PP2, had the opposite effect, namely inhibition of differentiation, as shown in Fig. 1C.

3.2. Effect of GA on PC12 cells

When PC12 cells were treated with GA, apoptosis was induced (Fig. 2). Again, this effect was not due to src inhibition,

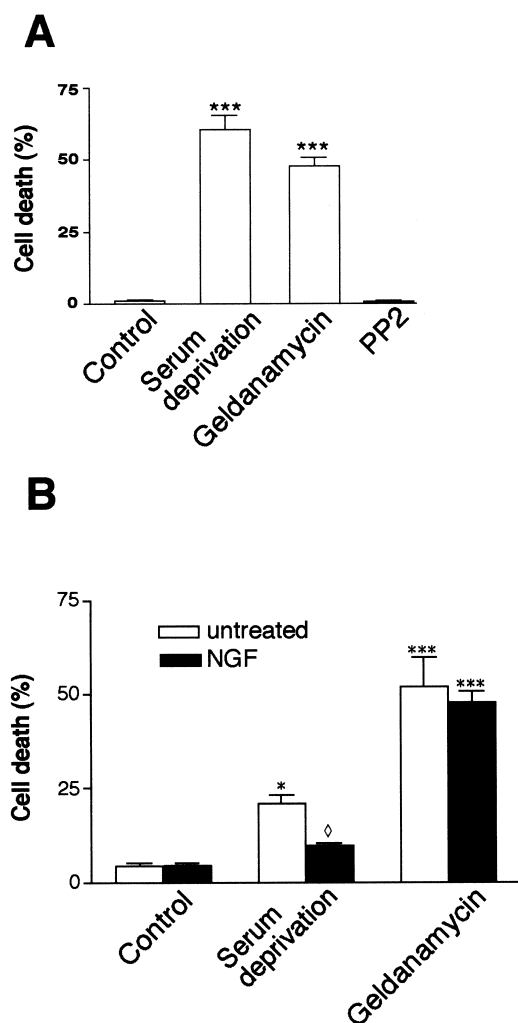


Fig. 2. Effect of GA on PC12 cells. Cells were, deprived of serum, treated with 2 μ M GA or with PP2 (5 μ M) for 48 h in the absence (open bars) or presence (filled bars) of 50 ng/ml NGF, and processed for flow cytometric analysis of apoptosis as described in Section 2. Data are expressed as mean $\pm$ standard error of three independent experiments. *** P < 0.001 with respect to control cells; $^{\diamond}$ P < 0.001 with respect to serum-deprived cells.

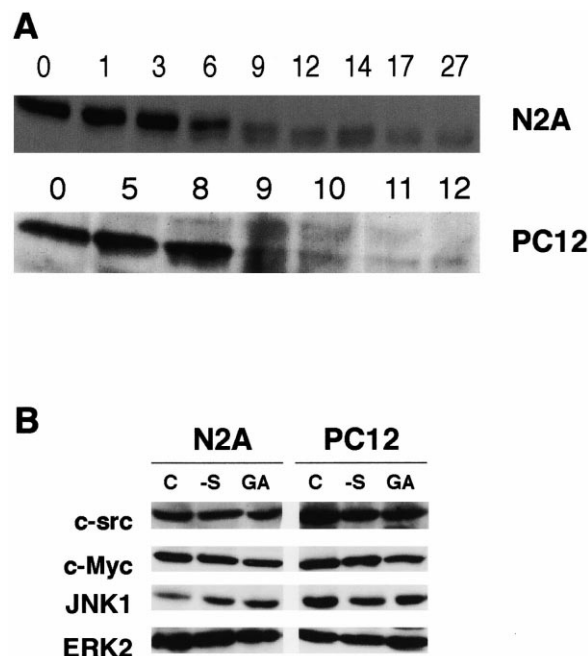


Fig. 3. Steady-state levels of Raf-1 and other proteins in GA treated N2A and PC12 cells. A: Disappearance of Raf-1 after treatment with GA. Individual cultures of N2A (above) or PC12 (below) were treated with GA, lysed at the indicated times (h) and subjected to Western blot analysis. B: Levels of different proteins in cells treated with GA. Individual cultures of N2A (left) or PC12 (right) were deprived of serum or treated with GA and lysed after 24 h. Western blot analysis was carried out with antibodies against c-src, c-Myc, ERK2 and JNK1 (C, control cultures; -S, cultures deprived of serum; GA, cultures treated with 2 μ M GA).

as PP2 had no effect on the survival of the cells. We checked if NGF, a well known survival factor for PC12 cells [23], could protect them from apoptosis induced by GA; as shown in Fig. 2, this is not the case; NGF was unable to induce survival in the presence of GA, although it was effective against apoptosis induced by serum withdrawal.

3.3. GA induces destabilization of Raf-1

As mentioned before, there are several reports that show that GA treatment destabilizes Raf-1 kinase, allowing its degradation by the proteasome [20]. Fig. 3A shows that Raf-1 disappears upon GA treatment in both cell types after approximately 9 h of treatment. This effect was specific for Raf-1, as other important signaling components, like c-src, c-Myc, ERK2 or JNK1 were not affected by treating the cells with the drug, as shown in Fig. 3B.

3.4. Mitogen-activated protein kinase (MAPK) pathways are affected by GA

It has been proposed for PC12 cells that sustained activation of ERK is sufficient for differentiation [2,3], whereas activation of JNK and inhibition of ERK are needed for apoptosis triggered by serum withdrawal [24]. We have studied, therefore, the effect of GA treatment on the activity of these kinases in N2A and PC12 cells. Fig. 4A shows that, in N2A cells, ERK activity raises during GA treatment, reaching maximum levels around 8 or 9 h after addition of the drug. In the same experiment JNK levels did not change significantly during the treatment. These results correlate with those obtained when N2A cells were deprived of serum, a differentiation sig-

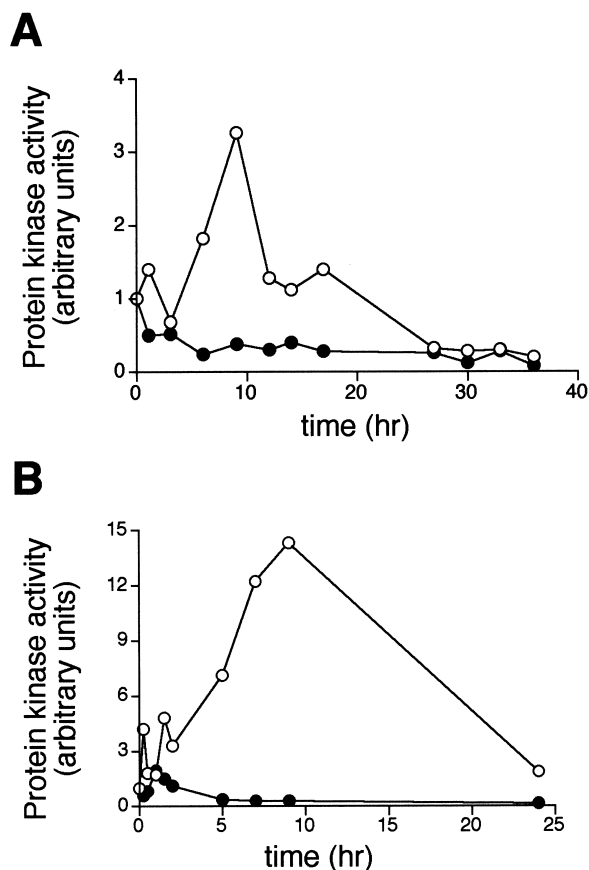


Fig. 4. Kinetics of ERK2 (open symbols) or JNK1 (closed symbols) activation in N2A cells after treatment with 2 μ M GA in N2A (A) or serum deprivation (B). ERK2 and JNK1 activities were assayed as described in Section 2. Activity of both enzymes at time zero was arbitrarily set to 1.

nal for these cells [25], as shown in Fig. 4B; ERK activity has a steady increase at approximately the same time as with the treatment with GA, and JNK has a small and transient peak of activation. At least in the case of serum deprivation, the sustained ERK activation seems to be determinant for the differentiation process, since inhibition of the pathway with the MEK inhibitor PD98059 [26] diminished differentiation by 50% (not shown).

The opposite effect was observed in PC12 cells; JNK was activated and reached a maximum at around 12 h of treatment, whereas ERK activity decreased below the basal levels found in untreated cells, with a similar kinetics of that observed for Raf disappearance (Fig. 5A). When cells were pretreated with the JNK inhibitor SB203580 [27] before adding GA, the induction of apoptosis was completely abolished (Fig. 5B), demonstrating the involvement of JNK in the induction of apoptosis.

4. Discussion

The results presented in this report show that different cell lines respond in different ways to the inhibition of Hsp90 by GA. Differences in response to GA, as those that we observe, have already been pointed out at an organismal level by Ruthford and Lindsquit [15]. These authors proposed Hsp90 as an evolutionary capacitor, acting as a buffer for avoiding ex-

pression of deleterious mutations in normal growth conditions. Should a stress situation take place, the canalization of Hsp90 to other roles instead of maintaining crucial signaling pathways properly, could permit rapid and profound changes in relatively short times. A cellular correlate of this scenario is shown by the different phenotypes that Hsp90 inhibition by GA can trigger in different cell lines.

We chose two cell lines that are neuronal model systems, and that behave differently upon serum deprivation; while N2A cells differentiate [25], PC12 cells enter in apoptosis [24,28]. N2A cell differentiation induced by withdrawal of serum resulted in activation of ERK activity, and the same is true for GA treatment of these cells. Our results of Raf-1 levels and ERK activity are consistent in the sense that we

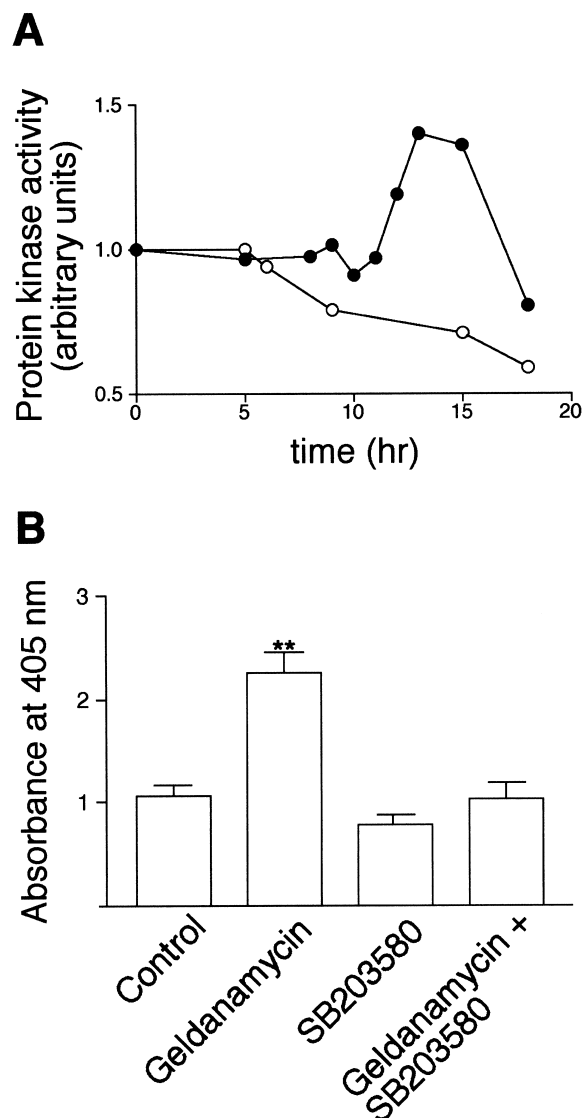


Fig. 5. Kinetics of ERK2 (open symbols) or JNK1 (closed symbols) activation in PC12 cells after treatment with 2 μ M GA (A). Suppression of apoptosis induced by GA (2 μ M) by pretreatment with SB203580 (25 μ M), in PC12 cells (B). Cells were treated for 24 h as shown in the figure and processed to measure apoptosis with an ELISA detection kit as described in Section 2. Absorbance at 405 nm measures cytoplasmic, histone-associated low-molecular weight DNA. Data are expressed as mean $\pm$ standard error of three independent experiments. ** P < 0.01 with respect to control cells.

observe activity while Raf-1 protein is present, as has been observed by others [29]. The problem of how ERK is activated by a process that ultimately destabilizes Raf-1 is not solved, although the lack of interaction between Raf-1 and Hsp90 could allow transient oligomerization and/or membrane translocation, resulting in activation of Raf-1 before its degradation. In fact, Hall-Jackson et al. have described an equivalent situation with the p38/MAPK inhibitor SB203580 [30].

Incubation of PC12 cells with GA induced of apoptosis, in a manner dependent of JNK activity activation, as shown by the fact that JNK inhibition results in survival of the cells. The timing of activation of JNK, 3–4 h after Raf-1 disappearance, is consistent with being this disappearance the trigger of apoptosis. The fact that NGF does not protect the cells from apoptosis poses the question of the ultimate trigger of death. NGF mediates its action through the TrkA receptor and the Ras/ERK signaling cascade, although signaling through the phosphatidylinositol-3'-kinase (PI3K) pathway have also been implicated [31]. The fact that GA-induced apoptosis is dominant over NGF protection could mean that both pathways, Ras- and PI3K-mediated, are simultaneously needed for survival of PC12 cells, or else, that although no activation of ERK would be needed for survival, some other Raf-1-dependent mechanism should exist.

It has been described that MEKK1, a MAP kinase kinase parallel to Raf-1, can activate both ERK and JNK pathways, interacting with MEK1 [32] and Ras [33] on one side, and SEK (the upstream kinase of JNK) [34,35] on the other. The GA effects we describe, activation of only ERK in N2A cells and only JNK in PC12 cells, do not seem to be related to MEKK1, as we should find activation of both pathways in both cell lines.

Although Raf-1 steady-state level modulation by inhibition of Hsp90 could account for the effects we observe, we have also searched for possible effects due to src protein kinase. While v-src is much more sensitive to Hsp90 inhibition, recent results show that this chaperone is also needed for proper folding of c-src [10]. As our results show, however, c-src is not involved in the observed phenotypes; since it has the opposite effect in N2A cells (inhibition of morphological differentiation), and has no effect in PC12 cells.

Note: When this manuscript was ready for submission, it came to our attention that GA also induces apoptosis due to Raf-1 destabilization in human luteinized granulosa cells [36].

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