Journal of Cellular Biochemistry 103:1005–1012 (2008)

Anisomycin Abrogates Repression of Protooncogene *c-fos* Transcription in E1A + cHa-ras-Transformed Cells Through Activation of MEK/ERK Kinase Cascade

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Abstract We have previously shown that transcription of immediate-early *c-fos* protooncogene is becoming strongly repressed in rat embryo fibroblasts transformed by oncogenes *E1A* and *cHa-ras*, so that serum only slightly stimulated *c-fos* transcription in these cells in contrast to high level of *c-fos* activation in non-transformed REF52 cells. Here we showed that stress-inducing agent anisomycin was able to override the *c-fos* repression and to induce *c-fos* transcription in E1A + ras transformants. In vitro kinase assay data demonstrated that anisomycin increased phosphorylation of transactivation domain of Elk-1 transcription factor—a key regulator of inducible *c-fos* transcription. Importantly, this activation was mediated through up-regulation of MEK/ERK but not stress-kinase cascades JNK or p38. The activating effect of anisomycin on *c-fos* transcription could be abrogated by a prior treatment with N-acetyl-L-cysteine. This indicates that anisomycin potentiates generation of reactive oxygen species (ROS), which, in turn, can modulate the activity of MAP kinase-specific phosphatases (MKPs). As anisomycin did not cause acetylation of nucleosome core histones, the present work focuses on the molecular mechanisms mediating the HDAC-independent induction of IEG *c-fos* by anisomycin in E1A + cHa-ras-transformed fibroblasts. J. Cell. Biochem. 103: 1005–1012, 2008.

Key words: MEK/ERK kinases; anisomycin; protooncogene *c-fos*; *E1A* and *cHa-ras* oncogenes

INTRODUCTION

Immediate-early response genes (IEGs), in particular protooncogene *c-fos*, undergo rapid and substantial transcriptional induction following growth factors addition and stress factor treatment. The protooncogene *c-fos* gene plays an important role in regulation of various target genes as encodes for a protein subunit of the transcription complex AP-1. In case of the certain mutations or over-expression, oncogene *c-fos* as well as its viral homologue *v-fos* can promote development of tumor cells [Saez et al., 1995; Ordway et al., 2004]. Signal transduction from the cell membrane to chromatin-regulated transcriptional machinery is provided by a number of protein kinase cascades. Of particular importance among them are the MEK/ERK cascade induced mainly by growth factors and the SAPK/JNK and p38 cascades that are activated by stress factors, such as ultraviolet light, antibiotics (anisomycin), xenobiotics (sodium arsenite), oxidative, and osmotic stresses [Treisman, 1996; Wilkinson and Millar, 2000]. Although anisomycin at low sublethal concentrations is shown to strongly activate the stress-dependent JNK and p38 kinase cascades [Cano et al., 1994; Hazzalin et al., 1998], there are data indicating that anisomycin is also able to induce the MEK/ERK cascade in a number of transformed cells [Torocsik and Szebereny, 2000; Bebien et al., 2003]. Activated ERK, JNK, and p38 kinases phosphorylate downstream effector kinases MSK, RSK, and MNK [Thomson et al., 1999] which, in turn, phosphorylate such transcription factors as TCF, SRF, CREB, ATF1, Elk-1 and core histones (H3) and non-histone proteins (HMG-14) [Thomson et al., 1999; Bebien et al., 2003; Soloaga et al., 2003].

Grant sponsor: Russian Foundation for Basic Research (RFBR); Grant numbers: 06-04-49261, 06-04-49058; Grant sponsor: CRDF-RFBR; Grant number: RUB1-2868-ST-07.

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Received 9 April 2007; Accepted 11 June 2007
DOI 10.1002/jcb.21471

Phosphorylation and acetylation of core histones promote formation of a relaxed chromatin structure [Thomson et al., 1999; Li et al., 2003], while phosphorylation of transcription factors interacting with promoter elements SRE and CRE/AP-1 leads to transactivation of IEGs *c-fos* and *c-jun* and other members of the AP-1 gene family [Hazzalin et al., 1998; Torocsik and Szebereny, 2000; Bebien et al., 2003; Li et al., 2003].

The current work was carried out on E1A+c-Ha-ras-transformed cells obtained by stable transfection of *E1A* and *cHa-ras* oncogenes in primary rat embryo fibroblasts (REF) [Pospelova et al., 1990]. These cells, due to permanent expression of E1A and Ras oncoproteins, have completely transformed status and proliferate at a high rate in the absence of growth factors and reveal constitutive activity of MAPK cascades [Pospelova et al., 1990; Kukushkin et al., 2002]. Elevated activity of MAPK cascades in E1A + ras cells leads to high and constitutive DNA-binding activity of transcription factors CREB, SRF, TCF, and AP-1, the latter being represented mainly by heterodimers Fra-1/c-Jun and ATF2/c-Jun [Pospelova et al., 1999; Kukushkin et al., 2002]. We have previously shown that due to constitutive activity of MAP kinases and abovementioned transcription factors the transcription of IEGs is also deregulated: while *c-jun* and *Egr-1* genes are transcribed at a high level, the transcription of *c-fos* gene is strongly repressed [Abramova et al., 2003]. According to our and other early data, the *c-fos* gene repression is predominantly provided by a recruitment of histone deacetylase (HDAC) activity to Elk-1 transcription factor followed by deacetylation of nucleosomal histones at the *c-fos* promoter [Yang et al., 2001; Kukushkin et al., 2002]. Correspondingly, the negative control of *c-fos* transcription can be relieved after treatment of E1A + ras transformants with HDAC inhibitors (sodium butyrate or valproic acid), which induce hyperacetylation of core histones and increase the *c-fos* transcription [Kukushkin et al., 2002; Usenko et al., 2003]. Here, we show that anisomycin is able to induce significantly the *c-fos* protooncogene transcription in E1A + ras cells (without change of histone acetylation) through activation of ERK1/2 kinases. The present work focuses on the molecular mechanisms mediating induction of IEG *c-fos* by anisomycin in E1A + cHa-ras transformed fibroblasts.

MATERIALS AND METHODS

Cells

REF transformed by oncogenes *E1A* and *cHa-ras* (E1A + ras cell line) [Pospelova et al., 1999] were cultivated in DME medium with 10% FCS. After serum starvation for 24 h in medium containing 0.5% FCS, cells were treated with MAPK inhibitors (Calbiochem) and/or anisomycin (ICN, mainly 50 ng/ml of medium or sometimes 250 ng/ml) dissolved in DMSO (see Results and Discussion Section and Figure Legends). Mouse embryo fibroblasts (MEF) with genotypes *p38α−/−* and *JNK1/2−/−* transformed by oncogene *E1A* and *c-Ha-ras* have been kindly given by Dr. D. Bulavin and Dr. A. Brichkina (respectively).

Quantitative determination of superoxide anion (SOA) generated in living cells was performed by a modified method [Greiber et al., 1998] using 0.5 mM lucigenin as substrate and luminometer TD-20/20 (Turner Designs).

RT-PCR

Total cellular RNA was isolated by method described in Chomczynski and Sacchi [1987]. Reverse transcription (RT) and subsequent PCR procedure were performed as described in our previous work [Abramova et al., 2003]. Primers used for RT-PCR analysis of rat and mouse genes *c-fos* and *GAPDH* (internal control) were following: *c-fos*—5′-CTCTAGTGCCAACTTTATCC-3′/5′-CTTCAAGTTGATCTGTCTCC-3′ (PCR-product—356 bp); *GAPDH*—(5′-TGTGATGGGTGTGAACCACG-3′/5′-CCAGTGAGCTTCCCGTTCAG-3′) (PCR-product—297 bp). All RT-PCR experiments were repeated at least in duplicate. The PCR products were resolved electrophoretically in 2% agarose gel.

Immunoblotting

Cell lysates were obtained with RIPA buffer [DeSeau et al., 1987] containing PBS solution, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, protease inhibitors (1 mM PMSF, leupeptin, pepstatin A, aprotinin in concentration 10 μg/ml) and phosphatase inhibitors (1 mM sodium vanadate, 5 mM EGTA, 10 mM NaF). Total histones from isolated cell nuclei were obtained as described in Kukushkin et al. [2005] in presence of the before-mentioned inhibitors.

Protein concentration was determined by Bradford's [1976] method. Proteins of cell extracts were separated by electrophoresis in 10% PAGE–SDS (polyacrylamide gel with SDS), whereas histones—in 12.5% PAGE–SDS and transferred onto nitrocellulose membranes (Bio-Rad). Pre-stained protein set SeeBlue Plus2 (Invitrogen) was used as molecular weight standard. The proteins were visualized by using antibodies to kinases ERK (sc-94), pERK (sc-7383) and to histone H3 phosphorylated on serine-10 (sc-8656-R; Santa Cruz Biotech). Goat anti-rabbit IgG (GAR) and rabbit anti-mouse IgG (RAM; Sigma) conjugated with HRP were used as secondary antibodies. Proteins on membranes were detected by ECL method (SuperSignal West Femto Maximum, Pierce). Each immunoblotting analysis was repeated at least twice. For in vitro kinase assay, cell extracts were incubated overnight with antibodies to ERK kinases and immunocomplexes were bound to Protein A-Sepharose beads [Kukushkin et al., 2002]. The washed beads were used as source of the kinases to phosphorylate GST-Elk-C fusion protein as substrate.

RESULTS AND DISCUSSION

REF transformed by oncogenes *E1A* and *c-Ha-ras* reveal down-regulation of a number of IEGs, in particular, protooncogene *c-fos* as compared with non-transformed REF cells [Kessler et al., 1999; Kukushkin et al., 2002]. In serum-starved transformants, the level of *c-fos* expression is extremely low, whereas after treatment of serum-starved cells with anisomycin at concentration 50 ng/ml the *c-fos* transcription starts very fast and reaches maximum by 1 h after anisomycin addition followed by its decrease (Fig. 1A). Anisomycin increased phosphorylation of ERK1/2 and JNK1 kinases, but not of p38 kinase in E1A + ras transformants. These data concerning to phosphorylation status of JNK and p38 kinases in the presence of anisomycin have been presented in our preceding article [Kukushkin et al., 2005]. The kinetics of ERK1/2 phosphorylation is very rapid and peaks at 10 min after anisomycin treatment. One hour thereafter, this level decreases and reaches its initial value (Fig. 2A).

Given that anisomycin can activate a number of MAPKs in E1A + ras transformed cells, we looked at which MAP kinase cascades were responsible for augmentation of *c-fos* transcription. For this, we used the following specific inhibitors of protein kinase pathways: PD98059—for MEK kinases, SB203580—for p38 kinases, and SP600125—for JNK kinases. We treated E1A + ras cells with the inhibitors for 30 min prior addition of anisomycin (50 ng/ml, 1 h). As seen in Figure 2B, PD98059 inhibits the anisomycin-induced phosphorylation of MEK-dependent ERK1/2 kinases. The subsequent RT-PCR analysis has shown that only MEK/ERK inhibitor PD98059 was able to suppress significantly the anisomycin-induced transcription of *c-fos* gene (Fig. 1B). In contrast, inhibitors of both p38 and JNK kinase cascades did not affect noticeably anisomycin-induced transcription of *c-fos* (Fig. 1B). Thus, transcriptional activation of *c-fos* gene in response to anisomycin in E1A + ras transformants is mainly mediated through MEK/ERK kinase cascade.

To confirm the contribution of MEK/ERK cascade to anisomycin-induced *c-fos* transcription, we used the cell lines derived from MEF with knockout of stress-kinase genes *JNK1* and *JNK2* or *p38α* and transformed by oncogenes *E1A* and *cHa-ras*. While in control serum-starved mEras cells the basal level of *c-fos* transcription was very low (Fig. 1C, lane 1), there were no *c-fos* transcripts in serum-starved transformants lacking either *JNK1/2* or *p38α* genes (lanes 3 and 5). Nevertheless, anisomycin induced *c-fos* transcription in the parental mEras as well as in JNK1/2−/− and p38α−/− transformants (Fig. 1C, even lanes). Thus these data prove that JNK1/2 and p38α kinases are dispensable for transcriptional induction of protooncogene *c-fos* with anisomycin in rodent E1A + ras-transformed fibroblasts.

To study whether serum- and anisomycin-dependent activation of *c-fos* gene is mediated through common MEK/ERK pathway, we carried out RT-PCR analysis of *c-fos* expression upon successive treatment of E1A + ras cells by serum and anisomycin. When serum-starved E1A + ras cells are stimulated by 10% FCS, transcription of *c-fos* gene is activated with a maximum at 45 min (Fig. 3A, lanes 1 and 2), while 2 h later the transcription level declines markedly (lane 3) and returns to an initial value (a refractory state, lane 1). However, if the cells serum-stimulated for 2 h were additionally treated for 1 h with anisomycin, there was a significant enhancement of *c-fos* transcription

(Fig. 3A, lane 5). When serum-starved transformants were treated separately by serum, anisomycin or hydrogen peroxide (the latter is a powerful inducer of reactive oxygen species (ROS)) for 1 h, anisomycin was found to activate transcription of *c-fos* gene more strongly than 10% FCS or peroxide (Fig. 1D). We have previously shown that *c-fos* gene repression in serum-treated E1A + ras cells was mediated through HDAC-dependent mechanisms and therefore could be relieved by HDAC inhibitors [Kukushkin et al., 2002]. There is evidence for different mechanisms of *c-fos* activation by anisomycin and HDAC inhibitors. Firstly, anisomycin itself stimulates *c-fos* transcription to a greater extent than HDAC inhibitor sodium butyrate (Fig. 1E). Second, serum-starved cells treated with sodium butyrate alone demonstrate weaker *c-fos* activation than in a combination of sodium butyrate and anisomycin (Fig. 1E, lanes 3 and 4). Moreover, the combined treatment by sodium butyrate and anisomycin gives little but reproducible synergistic effect on *c-fos* transcription implying that these reagents mediate their activation rather through different mechanisms (Fig. 1E, compare lanes 2 and 4). Densitometry of electrophoretic bands to evaluate the intensities for *c-fos* transcripts values relatively to *GAPDH* compared to untreated serum-starved cells supports this conclusion (Fig. 1E). As anisomycin does not change the level of nucleosomal core histone acetylation [Kukushkin et al., 2005], we suggest that anisomycin exerts its activation effect

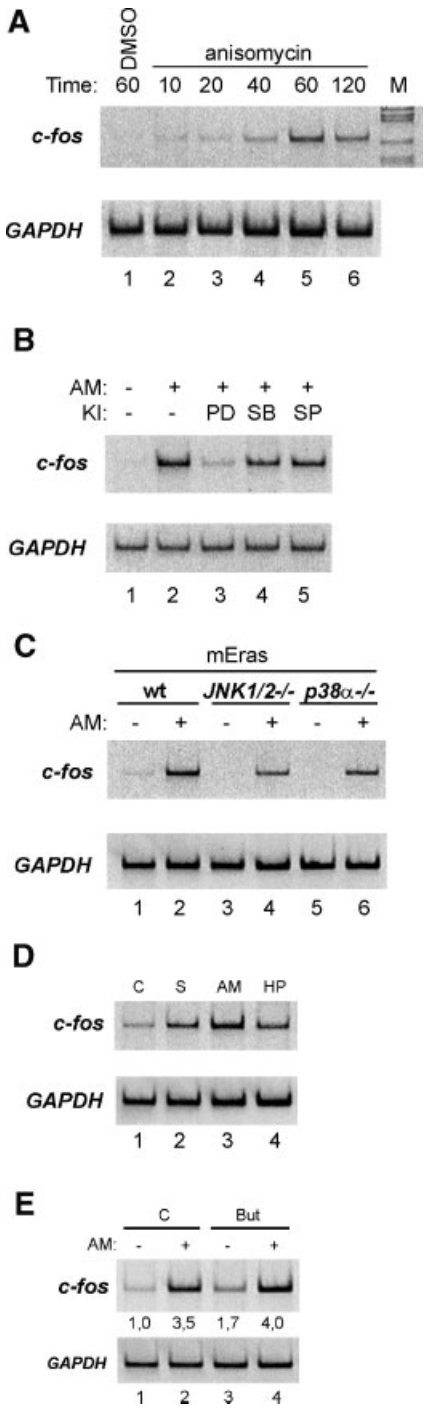

Fig. 1. Electrophoretic separation of RT-PCR products obtained with primers for cDNA of genes *c-fos* (the size of PCR-fragment is 356 bp) and *GAPDH* (297 bp) as an internal control in 2% agarose gel. M—DNA marker fragments. **A**: E1A + ras transformants serum-starved in DME medium with 0.5% FCS for 24 h were treated for 60 min either with DMSO alone (**lane 1**, control) or with anisomycin at concentration of 50 ng/ml for 10, 20, 40, 60, and 120 min (**lanes 2–6**, respectively). Total RNA was isolated and RT-PCR was performed. **B**: Serum-starved E1A + ras transformants were pre-treated for 1.5 h with DMSO (lanes 1 and 2) or with specific inhibitors of MAP-kinases (KI): 50 μM PD98059 (lane 3), 20 μM SB203580 (lane 4), 1 μM SP600125 (lane 5). After pre-treatment with MAPK inhibitors for 30 min, anisomycin (AM) was added for 1 h (AM, lanes 2–5). As a control (–), DMSO solvent of KI and AM was used. **C**: Three cell lines derived from MEF transformed by oncogenes *E1A* and *cHa-ras* (mEras: wild-type cells (wt), with knockout of *JNK1* and *JNK2* (*JNK1/2–/–*) or *p38α* (*p38α–/–*) genes) were serum-starved in DME medium with 0.5% FCS for 24 h and then treated for 60 min either with DMSO alone (lanes 1, 3, 5) or with anisomycin (AM) at concentration of 50 ng/ml (lanes 2, 4, 6). **D**: Serum-starved E1A + ras cells were treated for 1 h with DMSO (C, control lane 1), 10% FCS (S, lane 2), anisomycin (AM, 50 ng/ml, lanes 3), 0.2 mM hydrogen peroxide (HP, lane 4). **E**: Serum-starved E1A + ras cells were left untreated (C, lanes 1 and 2) or pre-treated for 6 h with 10 mM sodium butyrate (but, lanes 3 and 4). Then, to the end of incubation with butyrate, the cells were treated with DMSO (odd lanes) or anisomycin (AM, even lanes) for 60 min. The intensity values of *c-fos* transcripts relatively to control value in serum-starved cells (1.0 for lane 1) and of *GAPDH* (bottom panel) are shown between panels.

predominantly through a HDAC-independent pathway. One can assume that serum stimulation of *c-fos* gene encoding AP-1 factor subunit may mediate regulation of AP-1-dependent genes [Abramova et al., 2003] which are required for cell proliferation whereas anisomycin-induced transcriptional activation of *c-fos* and other IEGs may be important element of stress cellular response and be directed on a survival of cells [Roos and Kaina, 2006].

The effect of successive treatment with serum and anisomycin on the *c-fos* transcription is mediated through MEK/ERK cascade as it was shown above for anisomycin treatment alone (Fig. 2B). As expected, after serum stimulation ERK1/2 is moderately activated (Fig. 3B, lane 2), however the subsequent anisomycin treatment additionally increases ERK phosphorylation (lane 3). A prior treatment of serum-stimulated cells with MEK inhibitor PD98059 (30 min) eliminates completely the anisomycin-induced phosphorylation of ERK1/2. Moreover, the content of phosphorylated forms of ERKs decreases to the level comparable to serum-starved cells (Fig. 3B, lanes 4 and 1). Thus, although the content of ERK1/2 phosphorylated for tyrosine-204 is relatively high both in serum-starved and serum-stimulated E1A + ras transformants, anisomycin is capable of elevating the amount of phospho-ERKs. Hence, there is a clear correlation between the anisomycin-induced hyperphosphorylation of ERK1/2 kinases and transcriptional activation of *c-fos*.

Immunoprecipitation of ERK1/2 kinases followed by in vitro kinase assay with GST-Elk-C fusion protein as a substrate showed that immunoprecipitated ERKs from anisomycin-treated E1A + ras cells revealed an increased capability to phosphorylate the transactivation domain of Elk-1 factor (Fig. 3C). It is known that Elk-1 is a key factor for inducible regulation of the *c-fos* gene transcription [Whitmarsh et al., 1995; Yang et al., 2001; Kukushkin et al., 2002]. Pre-treatment with MEK inhibitor PD98059 suppressed the anisomycin-induced Elk-1 phosphorylation to a greater extent than inhibitors of JNK and p38 kinases that did not have such significant negative effect (Fig. 3C).

Stress factors are capable of activating specific protein kinase cascades and histone acetyl-

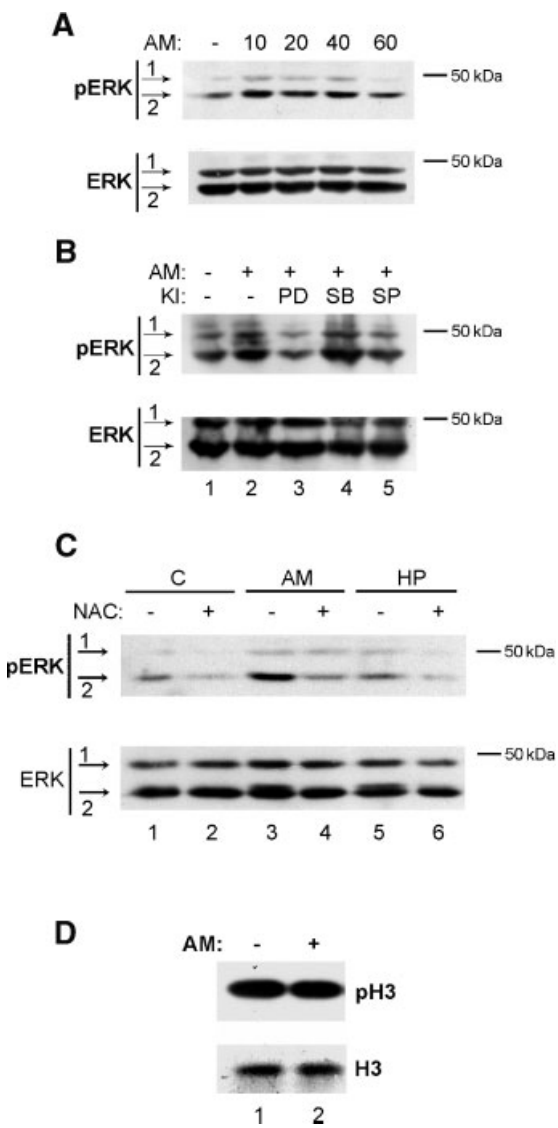

Fig. 2. **A–C**: Immunoblotting of protein kinases ERK1/2 from E1A + ras transformants treated with anisomycin (AM). Proteins of cell extracts were separated electrophoretically in 10% PAGE and transferred onto nitrocellulose membrane. Specific bands were revealed with antibodies to non-phosphorylated ERK1 and ERK2 kinases and to phosphorylated kinases pERK1, pERK2. Position of protein marker 50 kDa is indicated on the right. A: Cells after starvation were treated for 10 min with DMSO (−), lane 1 or AM at concentration of 50 ng/ml for 10, 20, 40, and 60 min (lanes 2–5, respectively). B: Designations are the same as in Figure 1B. C: Serum-starved E1A + ras cells were pre-treated for 4 h with 20 mM NAC (even lanes) or left-untreated (odd lanes), then they were incubated during 1 h with DMSO (C, lanes 1, 2) or with anisomycin (AM, lanes 3, 4) or with 0.2 mM hydrogen peroxide (HP, lanes 5, 6). **D**: Immunoblotting of histone H3 from E1A + ras transformants. Serum-starved E1A + ras cells were treated for 1 h with DMSO (control, lane 1) or anisomycin (AM, 250 ng/ml, lane 2). Total histones isolated from nuclei were separated electrophoretically in 12.5% PAGE–SDS. The membrane with transferred histones was incubated in the presence of antibodies to histone H3 phosphorylated on serine-10 (top) or total histone H3 was stained with Ponceau S on the membrane (bottom).

transferases that can lead to modifications (phosphorylation and acetylation) of nucleosomal histones thereby relaxing the chromatin structure [Clayton et al., 2000; Thomson et al., 2001; Li et al., 2003]. Recently, we have examined whether anisomycin could effect the histone acetylation in E1A + ras transformants and showed that low initial level of histone H3 acetylation on lysine-9 and lysine-14 was not changed in anisomycin-treated cells [Kukushkin et al., 2005]. On the contrary, the phosphorylation level of core histones is rather high in these cells, especially on serine-10 of histone H3; however, anisomycin remains the level also unchanged (Fig. 2D). As we fail to reveal a direct effect of anisomycin on acetylation and phosphorylation of core histones, we suggest that modulation of chromatin structure seems to be not involved in anisomycin-induced activation of *c-fos*. This means that anisomycin is capable of relieving negative regulation of *c-fos* gene transcription in E1A + ras cells by a mechanism which is different from the HDAC inhibitor-induced core histone acetylation. Interestingly, anisomycin does not change transcription of cyclin-kinase inhibitor $p21^{Waf1}$ gene (data not shown), which is up-regulated by various HDAC inhibitors [Abramova et al., 2006].

It was recently suggested that the anisomycin-induced hyperphosphorylation of ERK1/2 kinases could be caused by inhibition of phosphatases specific for ERK kinases (MKPs) [Brondello et al., 1997]. Published data [Torocsik and Szebereny, 2000] and our preliminary results indicate that anisomycin effect can be functionally linked with an increase of the

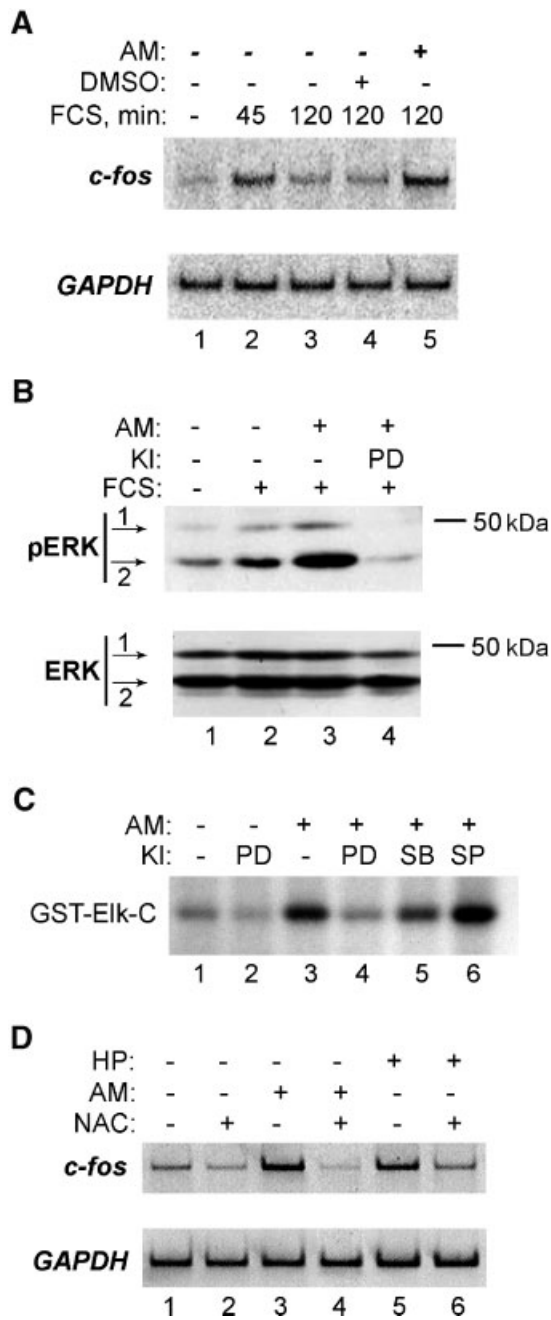

Fig. 3. **A**: Electrophoretic separation of RT-PCR products with primers for cDNA of genes *c-fos* and *GAPDH* in 2% agarose gel. E1A + ras cells were starved for 24 h in DME medium with 0.5% FCS (**lane 1**), then were stimulated with 10% FCS for 45 min (**lane 2**) or 120 min (**lanes 3–5**) followed by treatment for 60 min with DMSO (lane 4) or anisomycin (AM, lane 5). **B**: Immunoblotting of protein kinases ERK1 and ERK2 from E1A + ras cells after separation of proteins in 10% PAGE. After 24 h of starvation (lane 1), cells were stimulated with 10% FCS for 2 h (lanes 2–4) and 30 min after the start of serum stimulation, they were treated by DMSO (lane 3) or inhibitor PD98059 at concentration of 50 μM (lane 4) for 90 min. To the end of serum stimulation, cells were treated for 30 min with DMSO (lane 2) or anisomycin (AM, lanes 3 and 4). Proteins of interest were revealed by incubation of membranes with antibodies to non-phosphorylated kinases ERK and to their phosphorylated forms (pERK). Position of protein marker 50 kDa is indicated on the right. **C**: Analysis of activities of ERK kinases in vitro with GST-Elk-C as substrate. Lysates obtained from E1A + ras cells were immunoprecipitated with antibodies to ERK kinases. After 24 h of starvation, cells were treated for 1 h with DMSO (lanes 1 and 3) or with inhibitors of MAP kinases (KI): 50 μM PD98059 (lanes 2 and 4), 20 μM SB203580 (lane 5), and 1 μM SP600125 (lane 6). After 30 min, cells were treated for another 30 min with anisomycin (AM, lanes 3–6); in control (lanes 1 and 2) DMSO solvent of KI and AM was added. The washed ERK-immunocomplexes were used in reaction of in vitro phosphorylation with 1 μg of fusion protein GST-Elk-C. Protein products of phosphorylation were separated electrophoretically in 10% PAGE. **D**: Electrophoretic separation of RT-PCR products with primers for cDNA of genes *c-fos* and *GAPDH* in 2% agarose gel. Designations are the same as in Figure 2C.

TABLE I. Determination of Superoxide Anion Generation in Living E1A + ras Cells

Control (buffer without cells)	3.3 ± 1.3
E1A + ras cells, 0.5% FCS	37.9 ± 2.1
E1A + ras cells, 0.5% FCS, AM	44.3 ± 2.8

The serum-starved cells were treated with anisomycin (50 ng/ml) for 10 min. The reaction probes contained 5×10^5 washed cells, 0.5 mM lucigenin, Krebs buffer [Greiber et al., 1998] in volume of 0.1 ml. Control probes contained Krebs buffer. In given test, photon emission was counted for 0.5 min in 2 min after beginning of the reaction. Data are given as average values and standard deviations of four independent probes.

intracellular content of ROS. We have checked the content of SOA in preparations of living transformed cells using a photoemission reaction of SOA with lucigenin followed by photon counting in a luminometer [Greiber et al., 1998]. The appreciable SOA level was found in serum-starved E1A + ras cells in the presence of 0.5 mM lucigenin, but after short-term treatment with anisomycin (50 ng/ml, 10 min) the cells generated increased SOA level that was statistically valid in comparison with control probes (Table I). To illustrate possible role of ROS in anisomycin-induced effect, serum-starved E1A + ras transformants were pretreated for 4 h by N-acetyl-L-cysteine (NAC), a scavenger of ROS. Then, the cells were treated during 1 h with anisomycin or 0.2 mM hydrogen peroxide which was known as a source of ROS. Both anisomycin and peroxide caused strong activation of *c-fos* gene, while NAC pretreatment eliminated fully the activating effects of these stress factors (Fig. 3D). NAC treatment itself did not lead to transcriptional activation of *c-fos* (Fig. 3D, lane 2). Importantly, NAC pretreatment suppressed both anisomycin or peroxide-induced ERK1/2 phosphorylation and also basal phosphorylation of ERK kinases in serum-starved cells (Fig. 2C, top panel). Thus we suggest that anisomycin-induced ROS-mediated signals directed to transcription machinery of *c-fos* gene come through MEK/ERK cascade thereby allowing to abrogate repression of *c-fos* promoter. Among plethora of cellular effects of ROS, they can affect growth factor-independent proliferation of Ras-transformed cells [Irani et al., 1997]. Also ROS can oxidize functional cystein residues in active center of phosphatases [Finkel, 2000], thereby inactivating them and in one's turn contributing to maintenance of phosphorylation status of MAP kinases. Recently, the evidences in favor of this suggestion have been received for phosphatases of PP1, PP2A, and MKP groups [Kim et al., 2003]. In consistence with this, E1A + ras cells treated with okadaic acid, an inhibitor of serine phosphatases, demonstrated a remarkable augmentation of *c-fos* transcription [Kukushkin et al., 2002]. It is plausible that HDAC-dependent and ROS-mediated mechanisms can cooperate in process of regulation of a number of specific genes. Detailed analysis of the contribution of MAPK-specific phosphatases (MKP) to regulation of *c-fos* expression in anisomycin-treated E1A + cHa-ras transformed cells remains to be further studied.

ACKNOWLEDGMENTS

We are grateful to Dr. D. Bulavin and Dr. A. Brichkina for E1A + Ras-transformed mouse cells with deletions of *p38α* or *JNK1/2* genes.

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