Suppression of *c-fos* precursor RNA splicing by the protein kinase C inhibitor H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine]

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In JB6 epidermal cells, induction of *fos* proto-oncogene expression by phorbol 12-myristate 13-acetate can be inhibited by the protein kinase C (PKC) inhibitor H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine]. The compound causes also a dose-dependent suppression of *fos* precursor RNA splicing which, however, appears to react somewhat less sensitively to H7 than does PKC activity. This indicates that H7-induced accumulation of *fos* precursor RNA is not due to inhibition of PKC. Support for this interpretation comes from the finding that other inhibitors of PKC, such as *N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide dihydrochloride, sphingosine, staurosporine or *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide, do not suppress splicing when applied at PKC-inhibiting concentrations.

INTRODUCTION

Splicing of precursor RNA is an essential step in the expression of eukaryotic genetic information. Although it appears that this process is not the rate-limiting parameter in the generation of mRNA, it cannot be excluded that it is also subject to adaptative processes. Knowledge of the existence of respective regulating factors, therefore, would be of value in the understanding of mRNA formation.

The *c-fos* gene became a focal point of interest when it was found that the nuclear proto-oncogene functions as a transacting factor in conjunction with *jun* to mediate transcriptional activity of several genes through the consensus sequence TGACTCA [1]. Expression of *fos* can be induced by different agents which at least in part appear to mediate their effect by the activation of protein kinases such as cyclic AMP-dependent protein kinase A (PKA) and protein kinase C (PKC) [2–4]. In most cases this induction is effective only for a short time. The subsequent rapid down-regulation is controlled by the Fos protein itself [1]. Phorbol esters such as phorbol 12-myristate 13-acetate (PMA; 'TPA') activate PKC [5] and lead to a rapid induction of *fos* [3,6]. During our studies on the role of PKC down-regulation in promotion of JB6 epidermal cells to the tumour phenotype, we observed that the PKC inhibitor H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine] not only decreased PMA-induced *fos* mRNA formation, but also led to an accumulation of a new *fos*-specific RNA species. Here we described the characterization of this RNA as a *fos* precursor RNA, the conditions of its formation, and the specificity of H7 as an inducer of *fos* precursor RNA accumulation.

METHODS AND MATERIALS

JB6 Cl41 cells (7) were grown in Dulbecco's modified Eagle's medium containing 8% (v/v) fetal-calf serum (Boehringer, Mannheim, Germany). The inhibitors used were dissolved in appropriate solvents and added to confluent cultures for various times. Controls received solvents only. PMA (Sigma) and stauro-

sporine (Boehringer) were dissolved in acetone, H7 (Sigma), W7 [*N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide; Sigma] and HA1004 [*N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide dihydrochloride; Seikagaku Kogyo, Tokyo, Japan], in water, and sphingosine (Sigma) in water/acetone (1:1, v/v).

Total RNA was prepared from 2×10^7 cells in each experiment as described by Chirgwin [8] using CsCl-gradient centrifugation. From each preparation, 15 µg of RNA was separated in a 1% agarose gel and subjected to Northern blotting [9].

Fos probes were labelled with [α - 32 P]dATP (New England Nuclear Corp.) by nick translation [9], yielding specific radioactivities of $(1-2) \times 10^5$ c.p.m./ng of DNA. For RNA/DNA hybridization, filters were prehybridized overnight at 42 °C in a solution containing 25% (v/v) formamide, $6 \times$ SSC, $10 \times$ Denhardt's solution and tRNA (100 µg/ml). Hybridization of the specific probes was carried out in a solution containing 50% (v/v) formamide, $6 \times$ SSC, $10 \times$ Denhardt's solution, tRNA (100 µg/ml), 100 µM-dATP and 10 mM-sodium phosphate buffer, pH 6.8, over a period of up to 4 days at 42 °C. Washings were done with $6 \times$ SSC at room temperature, three times with $2 \times$ SSC/0.1% (w/v) SDS, three times with $0.2 \times$ SSC/0.1% (w/v) SDS at 55 °C and once with $0.2 \times$ SSC/0.1% (w/v) SDS at 65 °C. Kodak XAR5 films were exposed to the filters for up to 7 days. Autoradiographs were scanned using a transmittance/reflectance scanning densitometer model 1650 from Bio-Rad or a Camag TLC scanner II. Peaks corresponding to the strongest signal in each experiment were taken as 100%. The other signals are related to this arbitrary unit.

RESULTS

PKC is one of the governing mediators of *fos* expression in many mammalian cell systems, as discussed above. This is also true for JB6 epidermal cells, since expression of *fos* by PMA can be suppressed by various PKC inhibitors. The degree of inhibition follows their efficiency as suppressors of PKC activity *in vitro* (Fig. 1; cf. also [10]). Concentrations above IC_{50} (concn. causing

Abbreviations used: HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide dihydrochloride; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate ('TPA'); W7, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide; $1 \times$ SSC, 0.15 M-NaCl/0.015 M-sodium citrate; $1 \times$ Denhardt's solution, 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA; IC_{50} , concn. causing 50% inhibition; EC_{50} , concentration causing 50% of maximal effect.

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Fig. 1. Effect of various PKC inhibitors on PMA-induced *fos* expression

Control cells were treated with PMA (100 ng/ml) alone (●) and the experimental cells with PMA plus inhibitor (○). (a) 100 μ M-H7; (b) 160 μ M-HA1004; (c) 50 μ M-sphingosine (owing to the toxicity of sphingosine, the last points of the kinetic plot could not be evaluated); (d) 3.2 nM-staurosporine; (e) 20 μ M-W7; (f) 160 μ M-W7. β -Actin mRNA was not changed significantly in cells treated with PMA or with PMA plus inhibitor (results not shown).

Fig. 2. Transient accumulation of a 3.6 kb *fos* RNA in cells treated with PMA and H7

(a) Cells were treated with PMA (100 ng/ml) or (b) with PMA plus 100 μ M-H7. Hybridization with a *v-fos* probe was done as described in the Methods and materials section. Blots were exposed for 3 days. β -Actin mRNA was not changed significantly in cells treated with PMA, H7 or PMA plus H7 (results not shown).

Fig. 3. Kinetics of *fos* mRNA and precursor RNA induction by H7

The experiments were carried out as described in the legend to Fig. 2. Results are mean values from four experiments. Symbols: ●, 2.3 kb RNA after PMA treatment; ○, 2.3 kb RNA after PMA+H7; ▲, 3.6 kb RNA after PMA+H7 treatment. In cells treated with PMA alone, no 3.6 kb signal could be detected.

this kinase inhibitor was added at concentrations not affecting PKC (20 μ M; cf. [11], expression was higher than with PMA alone (Fig. 1e) suggesting that Ca^{2+} -calmodulin-dependent protein kinase exerts a negative control on the transcriptional activity of the *fos* gene [3]. However, when concentrations of W7 inhibitory to PKC [11] were applied, *fos* expression was completely blocked. Our data also show that the PMA effect does not involve co-operation of PKA, since HA1004, which preferentially suppresses PKA [4,12,13], inhibits *fos* expression only at concentrations $> \text{IC}_{50}$ for PKC, which is far above IC_{50} values for PKA.

H7 not only suppressed PMA-induced formation of *fos* mRNA but also, at the same time, led to the accumulation of a high-molecular-mass RNA species (Fig. 2). In addition to the *fos* messenger (≈ 2.3 kb), the applied *v-fos* probe detected the appearance of an RNA of 3.6 kb in cells treated with H7 and PMA. This size indicated that the additional RNA species represented the primary transcript of the *fos* gene [13], which cannot be seen in cells treated with PMA alone. The amount of *fos* precursor RNA normally found is about 1% of *fos* mRNA [14]. The kinetics of 2.3 kb *fos* RNA accumulation from several experiments were calculated from scans of the autoradiographs, and mean values are presented in relation to *fos* mRNA (Fig. 3). On average, maximal amounts were seen 30 min after the addition of PMA plus H7. Under these conditions, precursor RNA increased from less than 1% of the *fos* mRNA in PMA-treated cells [14] to about 50% in the presence of H7.

In order to confirm that the 3.6 kb band was indeed the primary transcript, hybridization was performed with a DNA fragment containing only the first intron of the *fos* gene (kindly provided by Professor R. Müller, Institut für Molekulare biologie und Tumor Forschung, Marburg, Germany). When total RNA from cells treated with PMA plus H7 was applied, this probe recognized only a 3.6 kb RNA (Fig. 4). No signal was detected in control cells or in cells treated with PMA or with H7 alone ((results not shown).

Accumulation of *fos* precursor RNA is dependent on H7 concentration (Fig. 5). Whereas amounts of *fos* mRNA decreased with increasing concentrations of H7, *fos* precursor RNA was augmented. At 300 μ M-H7, maximal amounts of precursor were observed. They even surpassed the amount of mRNA still present at this concentrations of the isoquinoline derivative. EC_{50} (concn. causing 50% of maximal effect) values for the two processes

50% inhibition) values led to a marked decrease in *fos* mRNA in all cases. Involvement of PKC is especially indicated by the divergent action of W7 at low and high concentrations. When

Fig. 4. Detection of 2.6 kb RNA by a *fos* intron probe in JB6 cells treated with PMA and H7

Cells were treated with PMA (100 ng/ml) plus 100 μ M-H7. At the indicated times, total RNA was isolated and analysed with a *fos* probe specific for the first intron. The autoradiograph was exposed for 7 days.

Fig. 5. Accumulation of *fos* precursor RNA as induced by increasing concentrations of H7 in the presence of PMA

Symbols: ○, mRNA; ●, precursor RNA. Cells were treated for 45 min with PMA (100 ng/ml) plus H7 as indicated. Total RNA was analysed with the *v-fos* probe. Quantification of the autoradiograph was performed as described in the Methods and materials section. Mean values from two experiments are shown. One autoradiograph (7 days exposure) is shown in the inset.

were clearly different. If 300 μ M-H7 is taken as the maximum for precursor RNA accumulation, an EC_{50} value of about 90 μ M can be derived from the data in Fig. 5, whereas the IC_{50} for mRNA accumulation is three times lower.

Accumulation of precursor RNA was not the result of a super-induction, since *fos*-specific RNA (mRNA + precursor RNA) in PMA-plus-H7-treated cells was far lower than that in cultures treated with PMA alone. Our findings thus indicate that H7 inhibits *fos* precursor splicing. Furthermore, as β -actin precursor RNA does not accumulate under these conditions, H7 is not a general inhibitor of RNA splicing. The results described above also demonstrate that inhibition by H7 of PKC-mediated *fos*

induction and inhibition of *fos* precursor splicing are independent events, thereby indicating that the action of H7 on *fos* precursor RNA splicing is a process not involving PKC. This interpretation is strongly supported by the observation (Fig. 1) that none of the other PKC inhibitors studied was able to induce an accumulation of the 3.6 kb precursor RNA in conjunction with PMA.

DISCUSSION

Induction of Fos by PMA has been shown to be associated with PKC-mediated phosphorylation of various substrates (e.g. p80) [15] and the transcriptional activation of PMA-responsive elements [16]. This applies to JB6 epidermal cells as well, because inhibitors of PKC suppressed *fos* mRNA formation according to their efficiency toward PKC activity (Fig. 1). When probing the inhibitor H7, it became evident that, in JB6 cells, expression of Fos requires an additional factor which is involved in the splicing of *fos* precursor RNA. Since super-induction of *fos* could be excluded, and because the isoquinoline derivative induced a specific accumulation of *fos* precursor mRNA in PMA-treated cells that was not seen with other inhibitors of PKC, H7 seems to interfere with *fos* mRNA formation at two levels: (i) it reduces induction of *fos* mRNA, in common with the other inhibitors of PKC, by reducing PMA-induced PKC activity; (ii) it inhibits *fos* precursor RNA splicing because the precursor molecule is accumulated at higher concentrations of H7. Since H7 as well as a number of related isoquinoline derivatives act at the ATP site of various kinases [17], the factor appears to be a kinase specifically inhibited by H7, though to a lesser extent than is PKC. The system resembles that of 2-aminopurine, which, in MG63 osteosarcoma cells, inhibited serum-induced accumulation of *fos* mRNA [18]. Because a kinase phosphorylating chromatin-associated pp33 in PMA-treated C3H10T1/2 cells is sensitive to 2-aminopurine [19], it is possible that this pp33 kinase is responsible for the stimulation of *fos* precursor RNA splicing. In this case, pp33 kinase should be sensitive to H7 but not to the other PKC inhibitors.

Our data thus lend support to the interpretation that the efficient production of *fos* mRNA includes the stimulation of a post-transcriptional process (splicing) by phosphorylation of nuclear acceptor(s), such as pp33.

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