MOLECULAR AND CELLULAR BIOLOGY, Nov. 1988, p. 5052–5055
0270-7306/88/115052-04$02.00/0

Vol. 8, No. 11

Platelet-Derived Growth Factor Does Not Induce c-*fos* in NIH 3T3 Cells Expressing the EJ-*ras* Oncogene

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Received 22 February 1988/Accepted 8 August 1988

Platelet-derived growth factor (PDGF), the calcium ionophore A23187, and the tumor promoter phorbol myristate acetate stimulated c-*fos* mRNA levels in control NIH 3T3 cells. However, NIH 3T3 cells transformed by EJ-*ras* DNA transfection, which have diminished PDGF-stimulated phospholipase C activity, showed a 95% reduction in PDGF-stimulated c-*fos* mRNA levels. The responses to A23187 and phorbol myristate acetate were also attenuated, but not as severely as the PDGF-mediated induction. The reduction in PDGF-stimulated c-*fos* induction did not appear to be a general result of cellular transformation, since *src*-transformed NIH 3T3 cells displayed a strong PDGF-stimulated c-*fos* induction. Despite the reduction in PDGF-stimulated c-*fos* induction, EJ-*ras*-transformed cells still responded mitogenically to PDGF. These data suggest that the magnitude of c-*fos* induction cannot be directly correlated with PDGF-stimulated mitogenesis in EJ-*ras*-transformed NIH 3T3 cells.

The family of human H-, K-, and N-*ras* genes encodes a highly conserved 21,000-dalton protein (p21) (14, 38) that appears to be involved in the control of cell growth and differentiation (17, 27, 35). Certain mutated or activated *ras* genes are found in various human neoplasms (5, 11, 12, 22, 25, 30, 34, 41). The activated *ras* genes that code for p21 are capable of morphologically and tumorigenically transforming NIH 3T3 cells. Recent discoveries have led to the suggestion that the p21 *ras* protein and guanine nucleotide regulatory proteins (G proteins) have similar biochemical activities within the cell (18, 19, 23, 26, 37). Previous work from our laboratory has shown that two systems that are regulated by G proteins, adenylate cyclase and platelet-derived growth factor (PDGF)-stimulated phospholipase C (PLC), are inhibited in EJ-*ras*-transformed NIH 3T3 cells (2, 3, 39). This loss of PDGF-stimulated PLC activity results in a diminution of inositol trisphosphate (IP_3) synthesis, Ca^{2+} mobilization, and diacylglycerol (DAG) synthesis. Despite this reduced PLC activity, EJ-*ras*-transformed NIH 3T3 cells still display a mitogenic response when exposed to PDGF (2).

Activation of the PDGF receptor is also associated with the induction of the proto-oncogenes c-*fos* and c-*myc* (1, 24, 28). Several groups have suggested that PDGF-mediated activation of PLC and subsequent Ca^{2+} mobilization and activation of protein kinase C regulate the induction of c-*fos* and c-*myc* (6, 32). However, desensitization of the protein kinase C pathway abolishes phorbol myristate acetate (PMA)-induced c-*myc* induction and leads to a reduction in PDGF-stimulated c-*myc* expression, but does not alter PDGF-mediated mitogenesis (10). Because EJ-*ras*-transformed NIH 3T3 cells have markedly reduced PDGF-stimulatd PLC activity, yet respond mitogenically to PDGF, they are an excellent system in which to examine the relationship(s) between PDGF-stimulated PLC activation, c-*fos* induction, and mitogenesis.

NIH 3T3 mouse fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (EC-10). pEJ, which is a pBR322 derivative carrying the EJ human bladder carcinoma oncogene, was obtained from the American Type Culture Collection (Rockville, Md.). pSRA-2, a permuted clone of Rous sarcoma virus DNA, was obtained from M. Bishop (University of California at San Francisco). pUCNeo, a pBR322 derivative which carries the neomycin gene from Tn*5* and the long terminal repeat from the Harvey murine sarcoma virus, was used as a selectable gene in the transfection experiments. Cotransfections were performed by coprecipitating 0.5 to 5 μg of *ras*-containing plasmid DNA in a 12:1 molar ratio with pUCNeo and 10 μg of calf thymus carrier DNA per ml (5). The cloned pSRA-2 plasmid DNA was restricted with *Sal*I and ligated to form concatamers prior to transfection. Selection for cells expressing the neomycin gene was done in EC-10 containing 1 mg of G418 (geneticin) per ml. Expression of the EJ-*ras* gene in transfected cells was confirmed by immunoblotting as described previously (39). Cultures of pUCNeo control or pEJ-transfected cells were maintained in EC-10. After the cells reached approximately 70% confluency, the medium was replaced with Dulbecco modified Eagle medium containing 1 μg of insulin per ml, 5 μg of transferrin per ml, 0.5 μg of bovine serum albumin per ml, and 0.5% fetal calf serum for 18 to 48 h. Under these conditions, the control cells become quiescent and growth of the EJ-*ras*-transformed cells slows. A complete growth curve can be found in reference 2. The serum-starved cells were then treated with PDGF (10 ng/ml), the calcium ionophore A23187 (0.5 μM), or PMA (0.1 μM). At the end of the incubation period, the medium was removed and the monolayer was washed twice with cold phosphate-buffered saline, and total RNA was isolated by guanidine thiocyanate solubilization and centrifugation over a cesium chloride cushion (7, 20). The RNA was further purified by chloroform-butanol extraction and ethanol precipitation (7, 20, 40) and quantitated by determining the A_{260}. The purified RNA was then denatured with glyoxal and dimethyl sulfoxide, electrophoresed on a 1.5% agarose gel, transferred to a Nytran membrane (Schleicher & Schuell, Inc., Keene, N.H.), and hybridized to a radiolabeled c-*fos* hybridization probe, a 1.4-kilobase *Bam*HI-*Xba*I DNA fragment of mouse c-*fos* genomic DNA isolated from p19/1 (8, 16, 33). To ensure correct RNA quantitation, we also hybridized both control and EJ blots with an 18S ribosomal hybridization probe, a

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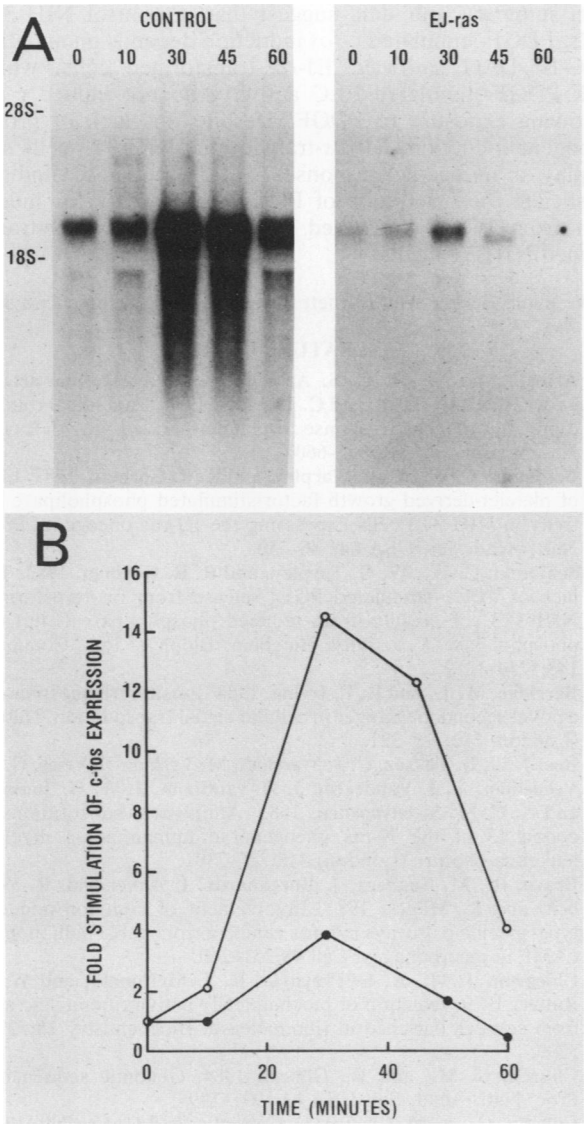

FIG. 1. PDGF-stimulated c-*fos* induction in control and EJ-*ras*-transformed cells. Control and EJ-*ras*-transformed NIH 3T3 cells were grown to 70% confluency in EC-10. The cells were then growth arrested by incubating them for 18 to 48 h in Dulbecco modified Eagle medium containing 1 μg of insulin per ml, 5 μg of transferrin per ml, 0.5 μg of bovine serum albumin per ml, and 0.5% fetal calf serum. The cells were then exposed to 10 ng of PDGF per ml for the indicted times (minutes), and mRNA levels were quantitated as described in the text. (A) Actual Northern blots; (B) densitometry of the spots. Each experiment was replicated at least three times.

1.1-kilobase *Bam*HI-*Eco*RI genomic DNA fragment isolated from pB (15).

Exposure of quiescent control NIH 3T3 cells to 10 ng of PDGF per ml resulted in a rapid but transient increase in c-*fos* mRNA levels. The actual Northern (RNA) blots are shown in Fig. 1A. The increase in c-*fos* mRNA was detectable at 10 min, peaked within 30 min, and decayed back toward basal levels by 60 min. A markedly different response was observed in NIH 3T3 cells expressing the EJ-*ras* oncogene. EJ-*ras* transformed cells had barely detectable basal levels of c-*fos* mRNA. The addition of 10 ng of PDGF per ml produced a very weak induction of c-*fos* mRNA. The time course of PDGF-stimulated c-*fos* induction in EJ-*ras*-transformed cells was essentially identical to the control time course, with a maximal response at 30 min (Fig. 1B). Identical results were obtained with cells maintained in 0.5% serum for up to 72 h. It should be emphasized that although PDGF produces only a weak induction of c-*fos* in EJ-*ras*-transformed cells, the transformed cells still respond mitogenically to PDGF. [^{3}H]thymidine incorporation was measured (31) in control and EJ-*ras*-transformed cells following exposure to 10 ng of PDGF per ml. Basal thymidine incorporation was 19,105 ± 2,162 and 24,557 ± 3,195 cpm/3 × 10^4 cells in control and EJ-*ras*-transformed cells, respectively. Following exposure of the cells to PDGF, these numbers were 160,572 ± 14,965 and 156,895 ± 11,505 cpm/3 × 10^4 cells, respectively. Thus, there was an 8.4-fold increase in thymidine incorporation in control cells and a 6.4-fold increase in EJ-*ras*-transformed cells. These data suggest that there is not a one-to-one relationship between PDGF-stimulated c-*fos* induction and PDGF-stimulated mitogenesis in EJ-*ras*-transformed cells.

In addition to PDGF, the calcium ionophore A23187 and the tumor promoter PMA also induced c-*fos* mRNA expression levels in NIH 3T3 cells. Presumably, these two agonists mimic IP_3 and DAG by mobilizing Ca^{2+} and activating protein kinase C, respectively. Figure 2A shows c-*fos* induction in control NIH 3T3 cells following exposure for 30 min to 10 ng of PDGF per ml, 0.5 μM A23187, or 0.1 μM PMA. There was an induction of c-*fos* mRNA with all three agonists. When this experiment was repeated with EJ-*ras*-transformed cells, a very different pattern emerged. PDGF caused a very weak induction of c-*fos*, whereas the effects of A23187 and PMA were somewhat stronger (Fig. 2A). However, it was clear from the Northern blots that the responses to PDGF, A23187, and PMA were all attenuated in EJ-*ras*-transformed cells. It should be noted that identical results were obtained from clonal lines of NIH 3T3 cells expressing EJ-*ras* p21. PDGF, A23187, and PMA do not induce c-*fos* (data not shown).

To assess the influence of the transformation process on PDGF-stimulated c-*fos* induction, we also exposed NIH 3T3 cells transformed by the v-*src* oncogene to 10 ng of PDGF per ml, 0.1 μM PMA, and 0.5 μM A23187 and measured mRNA levels by Northern analysis. *src*-transformed cells displayed a pattern of c-*fos* mRNA induction analogous to that observed in control cells (Fig. 2A). Although PDGF-stimulated c-*fos* induction in *src*-transformed cells was only about 60% as strong as that observed in control cells, it was at least an order of magnitude stronger than the response in EJ-*ras*-transformed cells (Fig. 2B). *src*-transformed cells also showed a much stronger c-*fos* induction in response to A23187 or PMA than EJ-*ras*-transformed cells (Fig. 2B).

After PDGF binds to its receptors on NIH 3T3 cells, it elicits a cascade of events which include the immediate autophosphorylation of the receptor and the activation of its intrinsic tyrosine kinase activity (13) and the degradation of phosphatidylinositol resulting in the production of DAG and IP_3. DAG leads to the activation of protein kinase C and subsequent phosphorylation of specific proteins, and IP_3 triggers the release of Ca^{2+} from intracellular stores (4, 29). Previous work from our laboratory showed that EJ-*ras*-transformed NIH 3T3 cells displayed reduced PDGF-stimulated PLC activity (2, 3). The data in this paper indicate that PDGF-stimulated c-*fos* induction is dampened in EJ-*ras*-transformed cells when compared with the response in control cells. In addition, the stimulation of c-*fos* induction by A23187 and PMA was also greater in control cells. These data suggest that although the loss in PDGF-stimulated PLC

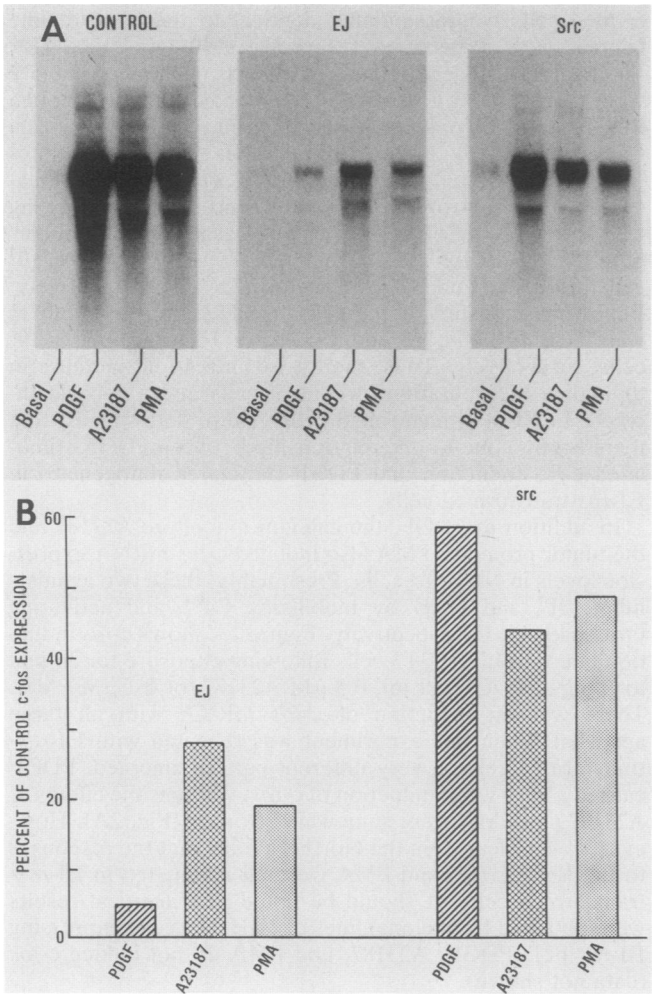

FIG. 2. Agonist-stimulated c-*fos* induction in control, EJ-*ras*-transformed, and *src*-transformed cells. The experiment was conducted exactly as described in the legend to Fig. 1. The cells were stimulated with 10 ng of PDGF per ml, 0.5 μM A23187, or 0.1 μM PMA for 30 min at 37°C, and mRNA levels were quantitated as described in the text. (A) Actual Northern blots; (B) Densitometry of the spots. Densitometry data are reported as a percentage of the corresponding response in control cells. Each experiment was replicated at least three times.

activation does contribute to the reduced c-*fos* induction, it does not account for all the reduction. Since both PMA- and A23187-stimulated c-*fos* inductions are also attenuated in EJ-*ras*-transformed cells, it is possible that mutated p21 also influences Ca^{2+} mobilization and protein kinase C activation as well as PLC. This overall dampening of c-*fos* induction in EJ-*ras*-transformed cells is not simply the result of the transformation process since v-*src*-transformed cells displayed strong c-*fos* induction to PDGF, A23187, and PMA.

It is not clear whether or not mutated *ras* blocks c-*fos* induction under all circumstances. Stacey et al. (36) recently reported similar serum-induced c-*fos* induction in control and *ras*-transformed cells. However, in support of our work, Colletta et al. (9) found attenuated thyrotropic hormone-, forskolin- and PMA-induced c-*fos* induction in FRTL-5 cells expressing v-*ras*. In addition, Guerrero et al. (21) have found that nerve growth factor does not induce c-*fos* or c-*myc* in PC-12 cells expressing high levels of N-*ras*.

In summary, our data suggest that in control NIH 3T3 cells, PDGF-stimulated c-*fos* induction depends on an intact PLC-IP_3-DAG pathway. EJ-*ras*-transformed cells, which lack PDGF-stimulated PLC activity, do not induce c-*fos* following exposure to PDGF. Despite this lack of proto-oncogene induction, EJ-*ras*-transformed NIH 3T3 cells still display a mitogenic response to PDGF. These findings dissociate the magnitude of PDGF-stimulated c-*fos* induction from PDGF-stimulated mitogenesis in EJ-*ras*-transformed NIH 3T3 cells.

We thank Bonnie-Ann Burnett for her valuable technical advice.

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