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

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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 (IP3) synthesis, Ca2+ 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 Ca2+ 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-stimulated 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 Tn5 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 SalI 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 (gentamicin) 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 using the A260. 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 BamHI-XhoI DNA fragment of mouse c-fos genomic DNA isolated from p191 (8, 16, 33). To ensure correct RNA quantitation, we also hybridized both control and EJ blots with an 18S ribosomal hybridization probe, a
formed 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. \[^3H\]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 cpn/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 cpn/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 \(\mu\)M A23187, or 0.1 \(\mu\)M 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 clones 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 \(\mu\)M PMA, and 0.5 \(\mu\)M 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. 2A). 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

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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 \(\mu\)g of insulin per ml, 5 \(\mu\)g of transferrin per ml, 0.5 \(\mu\)g 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 BamHI-EcoRI 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. \[^3H\]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 cpn/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 cpn/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.

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In summary, our data suggest that in control NIH 3T3 cells, PDGF-stimulated c-fos induction depends on an intact PLC-IP₃-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.

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LITERATURE CITED


