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Cell Biology

Persistent induction of cyclooxygenase in p60$^{v\text{-}src}$-transformed 3T3 fibroblasts

(prostaglandin endoperoxide synthase/Rous sarcoma virus/platelet-derived growth factor/protein kinase C/glucocorticoids)

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ABSTRACT **A BALB/c 3T3 cell line infected with the temperature-sensitive Rous sarcoma virus strain LA90 has been used to investigate early, p60$^{v\text{-}src}$-dependent changes in gene expression (protein synthesis). Giant two-dimensional electrophoresis, which can resolve >3000 polypeptides from [^{35}S]methionine-labeled cell lysates, was used to detect the induction of a p72-74 (72–74 kDa) doublet (pI 7.5) after activation of p60$^{v\text{-}src}$ at 35°C. Antiserum against cyclooxygenase (prostaglandin synthase or prostaglandin endoperoxide synthase) specifically immunoprecipitated the p72-74 doublet. The p72-74 doublet was also induced by platelet-derived growth factor and by phorbol 12-myristate 13-acetate and was elevated in an NIH 3T3 cell line transformed by wild-type *src*. Activation of p60$^{v\text{-}src}$ caused a persistant increase in p72-74, whereas the effect of the growth factor was transient. These dissimilar kinetics of induction were paralleled by changes in cyclooxygenase activity. Down-regulation of protein kinase C inhibited subsequent induction of cyclooxygenase by phorbol myristate acetate but did not block induction by p60$^{v\text{-}src}$. The glucocorticoid agonist dexamethasone inhibited induction of cyclooxygenase by p60$^{v\text{-}src}$. Although induction of this enzyme may not be directly involved in transformation, the data support the view that oncogenic transformation may result, not from expression of transformation-specific genes, but from persistent changes in the expression of genes normally induced only transiently during passage from the G_0 stage of the cell cycle.**

An important goal of studies of oncogene function is to identify gene products, the expression of which may be controlled by the oncogene of interest. Such identification might ultimately determine whether oncogenic transformation (*i*) is a consequence of the expression of transformation-specific genes, which are not normally expressed in growing cells, or rather (*ii*) is a result of the continuing expression at inappropriate times of genes normally induced only transiently during mitogenesis. One successful approach to this goal has been through differential and subtractive screening of cDNA libraries prepared from normal and oncogene-transformed cells (1), preferably using temperature-sensitive oncogene mutants; the latter enables the search for immediate-early genes that are induced rapidly rather than as the indirect end product of a cascade of events initiated by the oncogene. This approach has uncovered several interesting genes, the expression of which is altered in primary chicken embryo fibroblasts by the *src* oncogene (1–4). As an alternative approach to search for *src*-induced genes, we have exploited the high resolving power of giant two-dimensional electrophoresis (5) to look for rapid changes in protein abundance subsequent to the activation of p60$^{v\text{-}src}$ in a BALB/c 3T3 cell line infected with the Rous sarcoma virus temperature-sensitive mutant LA90 of *src*. The tyrosine-specific protein kinase activity of the LA90 p60$^{v\text{-}src}$ is inactive at 40°C but rapidly increases ($t_{1/2}$ = 5 min) when the cells are shifted to the permissive temperature of 35°C (6). One advantage of protein electrophoresis is that it can detect posttranscriptional changes in expression that might not be picked up by screening cDNA libraries. Surprisingly, however, of the >3000 polypeptides resolved by this method only two doublets exhibited prominent, reproducible increases. One of these doublets, of 72 to 74 kDa in size, has been identified as cyclooxygenase. The cyclooxygenase is also induced by platelet-derived growth factor (PDGF), but with different kinetics.

MATERIALS AND METHODS

Cell Lines. Rous sarcoma virus temperature-sensitive mutant LA90 (Schmidt–Rupin group D) was generated and initially characterized by Peter Vogt (University of California, Los Angeles) and used to infect BALB/c 3T3 fibroblasts (clone A31) by Joan Brugge (University of Pennsylvania). v-*src* transformed NIH 3T3 cells and their parental line were the gift of Mark Smith (National Cancer Institute–Frederick Cancer Research Facility).

Cell Culture and [^{35}S]Methionine Metabolic Labeling. The LA90 or A31 cells were grown to confluence in 2 ml of Dulbecco's modified Eagle's medium (DMEM)/10% calf serum in 35-mm culture plates at 40°C and starved in low-serum medium (1% calf serum) for 24 hr before stimulation with 10 ng of recombinant PDGF B (rPDGF-B) (p28$^{v\text{-}sis}$) per ml or by switching to 35°C for the indicated times. Cultures were then washed three times with methionine-free DMEM and labeled with [^{35}S]methionine (30-40 μCi/nmol; 1 Ci = 37 GBq) for 0.5–3 hr in DMEM/5 μM methionine/0.2% calf serum. The plates were then rinsed with ice-cold DMEM and extracted with 150–200 μl of lysis buffer (9.5 M urea/2% Nonidet 40/5% 2-mercaptoethanol/2% Ampholines).

Two-Dimensional Gel Electrophoresis. Details of procedures for giant two-dimensional gel electrophoresis have been published (5). Briefly, cell lysates containing 0.5–1 × 10^7 trichloroacetic-precipitable cpm were separated on tube gels using either isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGE). The second dimension used an $NaDodSO_4$ gel with a 10–16% exponential acrylamide gradient. Dried gels were exposed to Kodak XAR film for 6–12 days. Within an experiment, equal amounts of protein per sample were used, and differences in the amounts of acid-precipitable radioactivity were corrected for by adjusting the length of exposure of the gel to film. Molecular mass and pI of individual spots were estimated as

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis; PGE_2, prostaglandin E_2; PDGF, platelet-derived growth factor; rPDGF-B, recombinant PDGF (B-chain homodimer); PMA, phorbol 12-myristate 13-acetate.

described (7). Autoradiograms were analyzed by eye, and results were confirmed by densitometry with the microcomputer-based densitometer developed by Levenson *et al.* (8). Changes in autoradiographic density of proteins were scored as positive when they were present in all technically adequate autoradiograms at levels 3-fold higher (or lower) than the control.

Immunoprecipitation of [^{35}S]Methionine-Labeled Cyclooxygenase. Cells were solubilized in radio-immunoprecipitation (RIPA) buffer (9) after labeling with [^{35}S]methionine for 1 hr. Anti-cyclooxygenase antiserum or preimmune serum (10 μl) was then added to 500 μl of cell lysate, and the mixture was incubated for 3 hr at 4°C. Immune complexes were precipitated with *Staphylococcus aureus* suspension (100 μl) at 4°C for 1 hr, washed extensively with RIPA buffer, and then resuspended in the electrophoresis lysis buffer and analyzed by giant two-dimensional gel electrophoresis.

Assay of Cyclooxygenase Enzymatic Activity. Cells were rinsed with ice-cold phosphate-buffered saline, then solubilized by brief sonication in 50 mM Tris/1 mM diethyldithiocarbamic acid/10 mM EDTA/1% Nonidet P-40/α_2-macroglobulin at 0.2 mg/ml, pH 8.0, and assayed for cyclooxygenase activity by measuring conversion of added arachidonic acid to prostaglandin E_2 (PGE_2) as described (10). After addition of arachidonic acid (sodium salt solution, pH 9.0) to a final concentration of 100 μM, the reaction mixture was incubated for 30 min at 37°C. Aliquots of the incubation mixture were assayed for synthesis of PGE_2 by RIA (11).

Materials. Arachidonic acid was from Behring Diagnostics. Bicyclic PGE_2 assay system was from Amersham, dexamethasone was from Sigma, PDGF-B ($p28^{v\text{-}sis}$) was from Amgen Biologicals, and phorbol 12-myristate 13-acetate (PMA) was from LC Systems (Waltham, MA). [^{35}S]Methionine was from ICN, and the reagents for gel electrophoresis came from Bio-Rad and LKB. The rabbit anti-cyclooxygenase antiserum was a gift of David L. DeWitt and William L. Smith (Michigan State University, East Lansing). Characterization of this monospecific antiserum, which was prepared against purified cyclooxygenase from sheep vesicular gland, has been described (12).

RESULTS

$p60^{v\text{-}src}$-Early-Induced Proteins in LA90-Infected Cells. To examine early changes in the expression of specific gene products after $p60^{v\text{-}src}$ activation, LA90 BALB/c 3T3 cells were switched to the permissive temperature (35°C) for 3 hr, and cell lysates were submitted to giant two-dimensional gel electrophoresis. Of the >3000 ^{35}S-labeled polypeptides resolved by this method (9), surprisingly only two doublets (molecular size = 72 and 74 kDa, pI = 7.5; molecular size = 54 and 56 kDa, pI = 5.0) reproducibly increased in response to $p60^{v\text{-}src}$ activation. In this report attention is focused on the doublet shown in Fig. 1 (p72-74). The synthesis of p72-74 was enhanced not only by activation of the oncogenic $p60^{v\text{-}src}$ but also (at the nonpermissive temperature, 40°C) by rPDGF-B (Fig. 1 *A* and *C*) and a tumor-promoting phorbol ester (Fig. 1 *G* and *I*). The effect of activation of $p60^{v\text{-}src}$ on the changes in protein synthesis of p72-74 was not an artifact of the change in incubation temperature because the uninfected parental BALB/c 3T3 cells (A31 clone) synthesized a similar amount of p72-74 at 40°C and 35°C (Fig. 1 *D* and *E*). rPDGF-B also stimulates the synthesis of this doublet in A31 BALB/c 3T3 cells (Fig. 1*F*).

Identification of $p60^{v\text{-}src}$-Induced 72- and 74-kDa Protein Doublet as Cyclooxygenase by Immunoprecipitation. Similarities in molecular mass, isoelectric point, and subcellular distribution (unpublished data) between the $p60^{v\text{-}src}$-induced 72- and 74-kDa proteins and a previously described cyclooxygenase (10, 12, 13) suggested that this doublet was

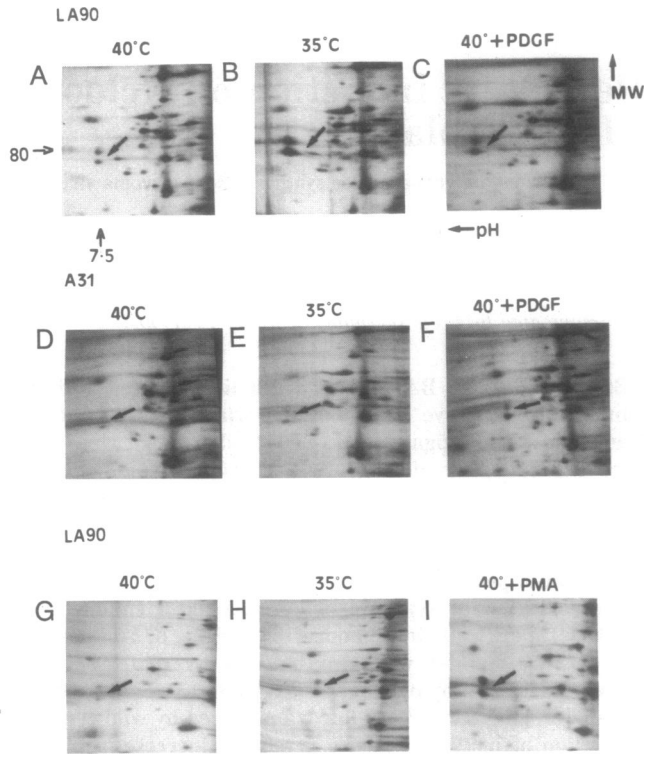

FIG. 1. Enhanced synthesis of a 72- to 74-kDa doublet by $p60^{v\text{-}src}$, PDGF, and PMA. Cells were starved by DMEM/1% calf serum at 40°C for 24 hr and then labeled with [^{35}S]methionine (30 μCi/nmol) for 3 hr in DMEM/5 μM methionine/0.2% calf serum under the conditions described below. Cells were then lysed and subjected to giant two-dimensional gel electrophoresis, as described. LA90-infected cells were either incubated at 40° (*A*) or 35°C (*B*) for 3 hr or were exposed to rPDGF-B ($p28^{v\text{-}sis}$; 10 ng/ml) for 3 hr at 40°C (*C*). The parental cell line of LA90 cells (clone A31) was tested at either 40°C (*D*) or 35°C (*E*) as control or was exposed to rPDGF-B (10 ng/ml) for 3 hr at 40°C (*F*). LA90-infected cells were incubated at 40°C for 2 hr (*G*) or 35°C for 2 hr (*H*) or treated with PMA (100 nM) for 2 hr at 40°C (*I*) and labeled with [^{35}S]methionine (40 μCi/nmol) for the final 30 min of incubation. All photographs represent at least three independent experiments, and each represents ≈5% of the total gel area (pH 7.0–7.7; 65–120 kDa). Arrows, p72-74 doublet. MW, M_r.

cyclooxygenase. From the many [^{35}S]methionine-labeled proteins in the cell lysates, a monospecific anticyclooxygenase antiserum selectively immunoprecipitated the $p60^{v\text{-}src}$-induced 72- and 74-kDa proteins and a series of related proteins on the gels (Fig. 2 *A* and *B*). In some experiments, the immunoprecipitates were less extensively washed so as to retain some of the abundant protein, actin (molecular mass = 43 kDa, pI = 5.6–5.7), for use as a marker in gel orientation (Fig. 2*A*). Fig. 2*B* shows that the amount of ^{35}S-labeled cyclooxygenase was significantly increased by shifting to the permissive temperature (35°C) and that rabbit preimmune serum did not immunoprecipitate any proteins from the LA90 cell lysates at either temperature. To confirm that the labeling increase was a result of increased synthesis rather than of a decrease in pI of a protein from beyond the pH range of the IEF gels, we also analyzed the immunoprecipitated material by NEPHGE (Fig. 2 *C* and *D*) and detected similar changes in labeling of the doublet. We conclude, therefore, that p72-74 represents murine cyclooxygenase and that it is rapidly induced in $p60^{v\text{-}src}$-transformed 3T3 fibroblasts.

Kinetics of Changes in Synthetic Rate and Cellular Enzymatic Activity of Cyclooxygenase Stimulated by $p60^{v\text{-}src}$ and PDGF. To determine whether any differences existed in the induction of cyclooxygenase by $p60^{v\text{-}src}$ and rPDGF-B, two parameters were measured in parallel: (*i*) the incorporation of

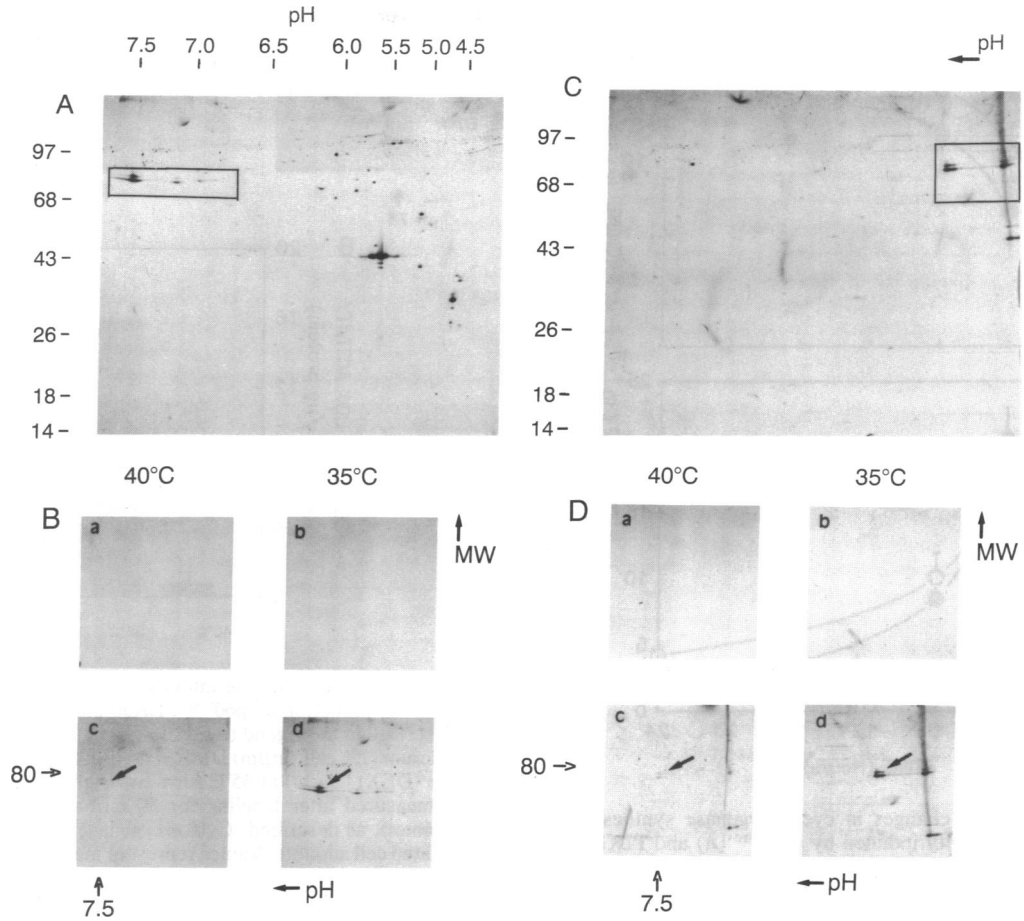

FIG. 2. Immunoprecipitation of the ^{35}S-labeled p72-74 doublet by anticyclooxygenase antiserum. LA90-infected cells were cultured in DMEM/1% calf serum for 24 hr and then incubated either at 40°C or 35°C for 24 hr in the same medium. Cells were then labeled with [^{35}S]methionine (40 μCi/nmol) for 1 hr, as described in the legend for Fig. 1. Cell lysates containing equal trichloroacetic-precipitable cpm were immunoprecipitated either by rabbit anticyclooxygenase antiserum [*A* (35°C); *B c* and *d*; *C* (35°C); *D*, *c* and *d*] or with preimmune serum *B*, *a* and *b*; *D*, *a* and *b*) and subjected to giant two-dimensional gel electrophoresis by using either equilibrium (IEF) separation (*A* and *B*) or NEPHGE separation (*C* and *D*) in the first dimension. Molecular size markers are in kDa. p72-74 doublet is boxed or indicated by arrows. MW, M_r.

^{35}S into p72-74 after short-term (30 min) labeling with [^{35}S]methionine, which provides a measure of the synthesis rate of the cyclooxygenase; and (*ii*) total cyclooxygenase enzymatic activity in solubilized cell sonicates. We found that the oncogenic protein p60$^{v\text{-}src}$ induced an early (1–2 hr) and persistent induction of cyclooxygenase (Fig. 3*A*), whereas rPDGF-B produced an early (1–2 hr) but transient increase that returned to baseline level after ≈8-hr treatment (Fig. 3*B*). The close parallel in the changes detected by the two assays suggests that the regulation of cyclooxygenase activity by p60$^{v\text{-}src}$ and PDGF occurs at the level of protein expression rather than through posttranslational effects.

p60$^{v\text{-}src}$ Induction of Cyclooxygenase Synthesis Is Independent of Activation of Protein Kinase C. PMA, a potent activator of protein kinase C (14), has been shown to induce cyclooxygenase in some human cells (15). To determine whether p60$^{v\text{-}src}$-induced cyclooxygenase synthesis depends on protein kinase C activation, advantage was taken of the ability of chronic phorbol ester administration to down-regulate the kinase and to suppress further stimulation by phorbol ester addition (16, 17). As shown in Table 1 (and Fig. 1 *G–I*), incubation with 100 nM PMA strongly stimulated the synthesis of cyclooxygenase (>10-fold) in LA90-infected cells at 40°C, as compared with a 4- to 5-fold induction by the activation of p60$^{v\text{-}src}$ for 2 hr. However, when the cells were pretreated with 500 nM PMA for 36 hr before challenge with PMA or switching the incubation temperature (Table 1), the effect of 100 nM PMA was inhibited (reduced to ≈4-fold), whereas the induction of cyclooxygenase synthesis by p60$^{v\text{-}src}$ was not significantly affected (4- to 5-fold induction). The down-regulation of protein kinase C after chronic exposure to PMA was confirmed from gel electrophoresis of lysates from ^{32}P-labeled cells (data not shown). Therefore, these experi-

Table 1. p60$^{v\text{-}src}$ induction of cyclooxygenase synthesis is independent of protein kinase C activation

Pretreatment	Treatment	Induction in ^{35}S-labeling, -fold
A 40°C	40°C	1.0 ± 0.2
B 40°C	35°C, 2 hr	5.0 ± 1.8
C 40°C	40°C, 100 nM PMA, 2 hr	12.5 ± 1.7
D 40°C, 500 nM PMA, 36 hr	40°C	1.0 ± 0.1
E 40°C, 500 nM PMA, 36 hr	35°C, 2 hr	5.0 ± 1.9
F 40°C, 500 nM PMA, 36 hr	40°C, 100 nM PMA, 2 hr	3.7 ± 1.7

LA90-infected cells were cultured as described in the legend for Fig. 1. Cells were treated as indicated above, labeled with [^{35}S]methionine (40 μCi/nmol) for the final 30 min of the incubations, and assayed as described. Values represent the means of -fold induction of radioactivity in [^{35}S]methionine (relative to 40°C, *A* or *D*) ± SEM. (n = 4). Intensities of p72-74 were derived by densitometric scanning of the lower spot on the doublet, as described (7), and values from each gel were normalized to the intensities of neighboring spots unaltered by the treatment.

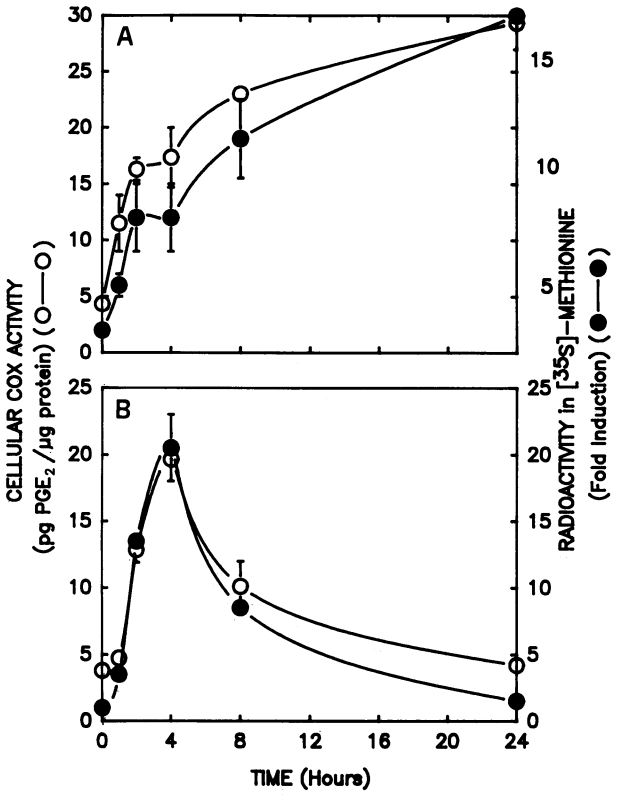

FIG. 3. Kinetics of changes in cyclooxygenase synthesis and cellular enzymatic activity induced by $p60^{v\text{-}src}$ (*A*) and PDGF (*B*). LA90 cells were cultured as described in the legend for Fig. 1. The cells were incubated at 35°C or treated at 40°C with rPDGF-B at 10 ng/ml for 0, 1, 2, 4, 8, or 24 hr in DMEM/1% calf serum and then labeled with [^{35}S]methionine (40 μCi/nmol) for the last 30 min of incubation as described and subjected to gel electrophoresis. Sonicated cell aliquots were assayed for cyclooxygenase (COX) activity by RIA as described. Values represent means ± SEM (n = 4). PGE_2, prostaglandin E.

ments provide evidence that $p60^{v\text{-}src}$ does not act through protein kinase C to stimulate cyclooxygenase synthesis.

Induction of Cyclooxygenase in v-*src*-Transformed NIH 3T3 Cells. To determine whether the induction of cyclooxygenase by $p60^{v\text{-}src}$ is an idiosyncracy of the LA90-infected BALB/c cells and whether it is expressed in constitutively transformed cell lines, we examined an NIH 3T3 fibroblast line that had been transformed by a wild-type v-*src* gene. Table 2 shows that both cyclooxygenase synthesis and enzymatic activity were significantly enhanced (≈2-fold) in v-*src*-transformed NIH 3T3 cells in comparison with their parental line—although to a lesser extent than in the LA90-infected cells. The smaller difference as compared with the BALB/c

Table 2. Expression of cyclooxygenase in *src*-transformed NIH 3T3 cells

Cell line	Cyclooxygenase activity, pg of PGE_2 per μg of protein	Induction in ^{35}S-labeling, -fold
NIH 3T3	4.0 ± 0.87	1.0
v-*src* 3T3	7.9 ± 1.96	2.24 ± 0.48

NIH 3T3 cells transformed by wild-type v-*src* and their parental line were cultured at 37°C, then labeled with [^{35}S]methionine (30 μCi/nmol) for 3 hr as described in the legend for Fig. 1 and subjected to giant two-dimensional gel electrophoresis and densitometric scanning (see text). Sonicated cell aliquots were assayed for cyclooxygenase activity as described. Values represent means ± SEM (n = 4).

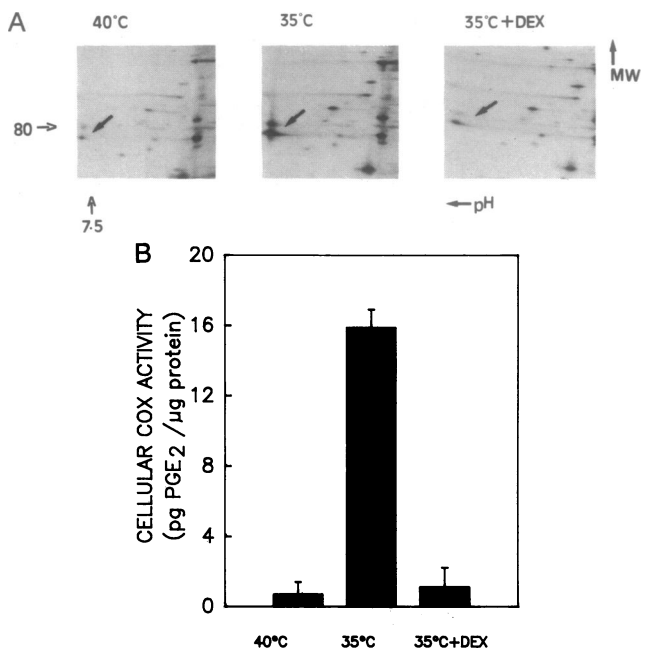

FIG. 4. Dexamethasone inhibits p72-74 (*A*) and cyclooxygenase activity (*B*) induction by $p60^{v\text{-}src}$. LA90-infected cells were cultured as described in the legend to Fig. 1. The cells were then incubated in low-serum (1% calf serum) DMEM medium alone or with dexamethasone (DEX) (2 μM) at 35°C for 8 hr. Synthesis of p72-74 (arrows) was measured after labeling for 30 min with [^{35}S]methionine (40 μCi/nmol), as described. Cyclooxygenase activity was assayed on sonicated cell aliquots. Values represent means ± SEM (n = 4). MW, M_r.

cells is, in part, a consequence of the 4-fold higher basal level of cyclooxygenase in the parental NIH 3T3 cells.

Inhibition of Cyclooxygenase Induction by Dexamethasone. The antiinflammatory glucocorticoid dexamethasone (2 μM) potently inhibited the induction by $p60^{v\text{-}src}$ of p72-74 synthesis and cyclooxygenase enzymatic activity in these LA90 cells (Fig. 4). Because dexamethasone causes partial reversion of some fibrosarcoma cell lines (18, 19), we asked whether dexamethasone or indomethacin (an inhibitor of cyclooxygenase) would affect the growth characteristics of LA90-infected cells. Neither drug inhibited the formation of foci in soft agar or cell growth nor inhibited the loss of contact inhibition at high cell densities exhibited by LA90 cells at the permissive temperature.

DISCUSSION

By giant two-dimensional electrophoresis and immunoprecipitation we have identified an immediate-early gene product, p72-74, as cyclooxygenase and have shown that this protein is persistently induced by $p60^{v\text{-}src}$, whereas PDGF stimulation results in a transient induction that subsides within ≈8 hr. Previous studies of *src*-transformed chicken embryo fibroblasts with similar techniques revealed many more changes in protein synthesis than reported here but did not detect p72-74 expression because the pH range used in the first dimension was slightly smaller (20). The larger number of changes is probably a consequence of the longer period (24 hr) during which the chicken cells were incubated at the permissive temperature (so that secondary changes in expression could have occurred) and the difference in the cell type. We have reported (21) that very few changes in protein phosphorylation accompany the activation of $p60^{v\text{-}src}$ in LA90-infected BALB/c 3T3 cells and that protein kinase C

is not activated, suggesting that only a subset of the total cellular response to *src* is expressed in these LA90 cells.

Interestingly, the immunoprecipitation revealed several spots concurrently induced by $p60^{v\text{-}src}$ and PDGF and which may represent posttranslationally modified forms of cyclooxygenase. The p72-74 doublet does not appear to arise because of phosphorylation and may be related to glycosylation (H.S. and D.A.Y., unpublished observations).

We have further shown that the total cellular cyclooxygenase activity correlates closely with the level of p72-74 synthesis, indicating that the primary regulation of cyclooxygenase is at the level of protein expression. Although PMA causes a large increase in p72-74 synthesis and factors such as interleukin 1 stimulate cyclooxygenase in other cell types by means of a protein kinase C-mediated pathway (13), the induction by $p60^{v\text{-}src}$ in LA90 BALB/c cells appears independent of the activation of protein kinase C. Expression of another v-*src*-induced gene, called *9E3* or CEF-4 (3, 4), on the other hand, has been reported to be regulated by means of the activation of protein kinase C, at least in chicken embryo fibroblasts (22).

Barker *et al.* (23) also have recently shown that the activation of $p60^{v\text{-}src}$ in chicken embryo fibroblasts increases the release of prostaglandins but suggested that the release originates upstream of phospholipase A_2 and the cyclooxygenase and may involve phospholipase C and protein kinase C-dependent pathways. Stimulation of phospholipase A_2, as measured by elevated levels of glycerophosphoinositol and nonesterified arachidonic acid, was also recently reported for NIH 3T3 cells transformed by *src* and other oncogenes (24). Whether these changes are causally related to transformation is unclear, but in the present study inhibition of p72-74 expression by glucocorticoid (dexamethasone)—which inhibits cyclooxygenase mRNA translation (13)—did not cause obvious reversion of the LA90 cells to the nontransformed state. Whether the expression of other $p60^{v\text{-}src}$-induced genes such as *9E3* (3, 4) is also extinguished by dexamethasone is an interesting question.

The level of cyclooxygenase protein is influenced in various cell and organ systems by steroids (25–27), growth factors (10, 26–31), and tumor promoters (15), suggesting that regulation of expression of this enzyme plays a role in the control of prostanoid formation. Induction is rapid (≈2 hr), but transient, occurring over a similar time course to other immediate-early genes, such as c-*fos* and c-*myc*. The major difference in the kinetics of expression in response to $p60^{v\text{-}src}$ is that induction by the oncogene is persistant, an effect also reported recently by Simmons *et al.* (1) for several other $p60^{v\text{-}src}$-induced immediate-early genes. The difference in response to mitogens leads us to suggest that oncogenic transformation might result, not from the expression of unique "transforming" genes, but from the persistant expression of genes normally expressed only transiently during passage from G_0 to G_1 stages of the cell cycle.

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