Dissociation of Platelet-derived Growth Factor (PDGF) Receptor Autophosphorylation from Other PDGF-mediated Second Messenger Events*

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Activated p21" alters the platelet-derived growth factor (PDGF) signal transduction pathway in fibroblasts by inhibiting autophosphorylation of the receptor as well as by inhibiting the induction of the growth-related genes c-myc, c-fos, and JE. To elucidate the cause and effect relationships between receptor autophosphorylation and other second messenger events in the PDGF signaling pathway we created revertants of v-ras transformed cells by two methods: 1) the use of cAMP analogues, and 2) the introduction of a gene, Krev-1, which has been reported previously to revert ras transformed cells to normal morphology. Analysis of the revertants shows that the PDGF-mediated tyrosine phosphorylation of the 180-kDa PDGF receptor remains inhibited; however, the PDGF-mediated activation of phospholipase C and the induction of the growth-related genes c-myc, c-fos, and JE have been restored. These data suggest the presence of parallel pathways for PDGF signal transduction which are not dependent on autophosphorylation of the PDGF receptor.

Growth factor signal transduction through the platelet-derived growth factor receptor consists of a complex cascade of events. The platelet-derived growth factor (PDGF) is a 30-kDa dimer that binds to the PDGF receptor, a 180-kDa glycoprotein homo- or heterodimer with at least two classes of subunits, α and β (Bowen-Pope et al., 1989; Claesson-Welch et al., 1988, 1989; Escobedo et al., 1988; Matsui et al., 1989; Siefert et al., 1989; Yarden et al., 1986). The PDGF receptor possesses an intrinsic protein tyrosine kinase activity that is stimulated upon binding by PDGF (Bowen-Pope et al., 1989; Heldin et al., 1989; Williams, 1989). A number of intracellular activities or events occur rapidly after exposure of a cell to PDGF, including dimerization and autophosphorylation of the PDGF receptor, association and activation of several proteins (including phospholipase C (PLC), phosphatidylinositol-3 kinase (PI-3 kinase), and Raf-1), increased phosphatidylinositol turnover and calcium mobilization, activation of protein kinase C, and induction of a number of growth-related genes, including c-myc, c-fos, JE, and c-jun (Kumjian et al., 1989; Meisshelder et al., 1989; Morrison et al., 1989; Wahl et al., 1989; Williams, 1989). A causal or sequential relationship of these PDGF-induced phenomena to each other and to eventual DNA synthesis is suspected but not well established.

The roles that cellular proto-oncogenes play in this cascade of events have also not been fully elucidated. One in particular, c-ras, may be implicated in PDGF signal transduction. Microinjection of anti-ras antibody arrests cells before they can enter S phase of DNA synthesis (Mulcahy et al., 1985). We and others have shown previously that mutated and activated ras genes (EJ-ras or Kirsten v-ras) block PDGF-mediated DNA synthesis in murine fibroblasts and mitogenic signal transduction in a number of other cell types (Zullo et al., 1988; Lichtman et al., 1986; Lichtman et al., 1987). Fibroblasts containing an activated ras gene demonstrate an inhibition of PDGF-induced autophosphorylation of its receptor (Rake et al., 1981), diminished PI turnover (Parries et al., 1987), diminished PLC activity (Benjamin et al., 1987), diminished calcium mobilization (Benjamin et al., 1988), and inhibited induction of c-myc, c-fos, and JE (Zullo and Faller, 1988). We have therefore utilized cells containing activated ras genes as "mutants" in PDGF signaling to study second messenger systems in the PDGF mitogenic pathway. The ability to create revertants of the ras transformed phenotype then permits determination of which of the phenomena associated with PDGF binding revert as well and may thus be causally related.

Exposure to cAMP phenotypically reverts ras transformed cells to a more normal morphology (Anderson et al., 1974; Carchman et al., 1974) and leads to partial return of PI turnover and calcium mobilization after PDGF stimulation (Olinger et al., 1989). Kitayama et al. (1989) have isolated a gene, Krev-1, which encodes a 21-kDa protein possessing homology to the ras family and which reverts ras transformed cells to a flat morphology. We created new revertants of Kirsten v-ras transformed Balb/c 3T3 cells (KBalb) by two methods: 1) treatment of KBalb cells with cAMP; or 2) transfecting the Krev-1 DNA into KBalb cells. The revertant cells produced by either method possess morphologic and growth characteristics similar to the untransformed Balb/c 3T3 (Balb) fibroblasts.
In the revertant cells partial reconstitution of the PDGF signaling pathway is demonstrated. In response to PDGF, increases in PLC activity as measured by phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis, mobilization of calcium, and the induction of c-myc, c-fos, and JE were restored whereas the PDGF-mediated autophosphorylation of the PDGF receptor remained blocked. These observations demonstrate a dissociation between autophosphorylation of the PDGF receptor and the activation of a number of presumed second messengers of the PDGF signaling pathway.

MATERIALS AND METHODS

Cells, Cell Lines, and Cyclic AMP Treatment—Balb/c 3T3 fibroblasts were obtained from the American Type Culture Collection. Kirsten v-ras transformed Balb/c 3T3 fibroblasts (KBalb) were created as described previously (Zullo and Faller, 1988). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% donor calf serum (GIBCO). The cell lines were treated with cyclic AMP by addition to the media of 2 mM N’-2’-O-dibutyryl-adenosine 3’5’-cyclic monophosphate (Sigma) for 48 h. As a control for the cyclic AMP studies, cells were also treated in 4 mM sodium butyrate to control the release into the cells of 2 nol of butyrate following cell death of cyclic AMP.

Transfection of Kreu-1 into KBalb—A plasmid containing both the Kreu-1 gene and the neo gene with each driven by an SV40 early promoter was the generous gift of Yoji Ikawa, Tsukuba Life Science Center, Japan. The plasmid was amplified by transformation into E. coli (Maniatis et al., 1983) plated on the wells to directly for electroporation into Balb and KBalb cells. After electroporation the cells were selected in Dulbecco’s modified Eagle’s medium containing 0.5 mg/ml G418 (Geneticin, GIBCO). After several weeks, isolated colonies containing clones of resistant cells were identified. Colonies with the flattest morphology were picked and expanded and then maintained in medium containing 0.5 mg/ml G418.

Cell Proliferation Assays—1 x 10° cells/well were plated in medium plus 10% donor calf serum into six-well tissue culture plates. To some wells dibutyryl cAMP was added to 2 mM. After 24 h the medium in some wells was changed to 0.5% serum. The number of cells in each well (in duplicate wells for each time point) was enumerated on days 0, 2, 4, and 6.

[3H]Thymidine Incorporation—5 x 10° cells/well were plated into 24-well tissue culture plates. At confluence cells were starved for 24 h in medium containing 0.5% donor calf serum, with some wells containing dibutyryl cAMP at 2 mM. After 24 h [3H]thymidine (Du Pont-New England Nuclear; specific activity, 20 Ci/mmol) was added to 5 Ci/ml. To some of the plates recombinant PDGF-BB (Amgen) or serum was added to final concentration of 10 ng/ml or 10%, respectively, and incubation at 37°C was continued. After 24 h plates were washed twice with cold phosphate-buffered saline, and Triton X-100 was added to 1% to lyse the cells. Nucleic acids were harvested onto glass fiber filters, and incorporated thymidine was quantitated by scintillation counting. Each cell type and condition was tested in quadruplicate.

Phospholipase C Activity—Phospholipase C activity was measured in quiescent cells or cells stimulated with 40 ng/ml PDGF-BB by quantitating PIP2 hydrolysis in the membrane fractions using the method of Jackowski et al. (1986). Each cell type was quantitated in quadruplicate.

Phospholipase C activity was also measured in anti-phosphoryto- sine immunoprecipitates of quiescent cells or cells stimulated with 40 ng/ml PDGF-BB, using the technique of Wahl et al. (1988). Phosphatidylinositol-3-Kinase Activity—Phosphatidylinositol-3 kinase activity was assayed in anti-phosphotyrosine immunoprecipitates of quiescent cells and cells stimulated with 40 ng/ml PDGF-BB as described elsewhere (Kaplan et al., 1986).

Calcium Mobilization—Cells were starved for 24 h in medium containing 0.5% donor calf serum. The spent medium was removed from the cells and saved. Cells were removed from the tissue culture plate with warm Versene (GIBCO), pelleted by centrifugation, resuspended in the spent medium, and incubated for 15–30 min at 37°C. The cells were then pelleted and resuspended in 106 cells/ml in a buffered assay solution containing glucose and 1 mM INDO-1, incubated at room temperature for 20 min to allow uptake of the INDO-1, and washed once in 50 volumes of balanced salt solution to remove excess dye. Calcium mobilization in response to recombinant PDGF-BB was then determined using a stirred cuvette at 37°C in an Aminco fluorometer with real-time recording at 405 and 480 nm.

Phospholipase C and Phorbol Ester Treatment of Balb and KBalb Cells—Phospholipase C (PLC) from Bacillus cereus was obtained as an enzyme preparation in 3.2 M guanidine-HCl, pH 6.0 (Boehringer Mannheim). The PLC was added to serum-starved cell cultures to a concentration of 2 units/ml. An equal volume of protein-free ammonium sulfate solution was added to control cultures. A final concentration of PLC greater than 2 units/ml or of ammonium sulfate greater than 45 mM was each toxic to the cells. Cells were treated for 60 min at 37°C, and RNA was extracted and analyzed to assess the response of the growth-related genes to the PLC treatment.

Phorbol 12-myristate 13-acetate (PMA) was added to serum-starved cells to a final concentration of 200 ng/ml and incubated for 60 min at 37°C. RNA was then isolated and transcripts quantitated by RNA blot analysis.

RNA Blot Analysis of Total Cellular RNA—Total cellular RNA was isolated by extraction with guanidine thiocyanate, separated on formaldehyde-agarose gels, and transferred to nitrocellulose or supported nitrocellulose membranes. RNA blot analysis was carried out as described previously (Offermann and Faller, 1988). Transcript levels were determined by hybridization of the autoradiographs with a Hewlett Packard ScanJet, Macintosh IIcx, Image 1.2.

[3H]DJNA Probes—32P-Labeled DNA probes were made by the random oligonucleotide primer method (Feinberg and Vogelstein, 1983). The probes specific for c-myc exon 2 and c-fos have been described previously (Faller et al., 1988). An equal volume of 0.75-kilobase PstI fragment containing exons 3 and 4. The ATPase-specific probe was a PstI 3.7-kilobase fragment of a rat sodium potassium ATPase α subunit cDNA (gift of Russell M. Medford, Emory University). Kreu-1 mRNA was detected with a 2.0-kilobase BamHI-PstI fragment of the Kreu-1 plasmid used for transfections.

Sulfonilized Cell Kinase Assay—The various cell lines were subjected to tyrosine kinase assay by solubilization of membrane proteins as described previously (Rakie et al., 1991). In brief, cells were grown to confluence and the media exchanged for methionine-free medium (GIBCO) containing [35S]methionine (Du Pont-New England Nuclear; specific activity, 44 Ci/mmol) for 1 h at 37°C. Cells were washed twice with phosphate-buffered saline, twice with the isotonic wash (120 mM KCl, 30 mM NaCl, 2 mM MnCl2, 10 mM HEPES, pH 7.4), overlaid with solubilization buffer (isotonic wash, 0.1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride) at 750 μl/100-mm plate, incubated at 0°C for 5 min, and then scraped into 15-ml conical tubes. An additional 750 μl was used to rinse the plate, and the nuclei were pelleted. The supernatant was kept on ice. A Bradford protein assay kit (Bio-Rad) was used to determine the protein concentration.

To 100 μg of solubilized protein were added 10 ng/ml recombinant PDGF-BB and 0.4 μM ATP, and the reaction was incubated for 5 min at 30°C. The reaction was quenched with ice, and 2 μl ATP buffer (1% Triton X-100, 10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM Na3PO4, 50 mM NaF, 0.1 mM NaVO3, 0.1% bovine serum albumin, 0.1% NaN3, 2 mM phenylmethylsulfonyl fluoride), and the result mixture was subjected directly to anti-phospho- tyrosine immunoprecipitation.

Diphospho-tyrosinylated Tyrosine Kinase Assay—In a modification of the technique described by Erusalimsky et al. (1999) cells were grown to confluence in 35-mm wells and then starved in 0.5% calf serum for 24 h. After washing twice with phosphate-buffered saline and twice with isotonic wash (as above) the cells were overlaid with a suspension of ammonium sulfate (45 mM) was each toxic to the cells. Cells were treated for 60 min at 37°C, and RNA was extracted and analyzed to assess the response of the growth-related genes to the PLC treatment.

Immunoblot Detection of PDGF Receptor-associated Tyrosine Phosphorylation—Cells were grown to confluence in 100-mm culture dishes. Monolayers were washed twice with phosphate-buffered saline, then 1 ml of HTG buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na2VO3) was added, the cells were dislodged with a cell scraper, the lysate was transferred to a microcentrifuge tube, cell debris was pelleted, and the specimens were normalized for protein content. The immunoblot was rechecked with 1 ml of a suspension consisting of 250 ng of beads/ml of HTG buffer for 1 h at 4°C, and 2 μl of antibody to PDGF type β

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receptor (gift of Tom Daniel, Vanderbilt University) was added to the mixture, which was rocked for 2 h at 4 °C. The receptor antibody complexes were precipitated by incubation with 100 μl of protein A-Sepharose for 1 h. The beads were heated to 100 °C in the presence of SDS, and equal protein amounts of the samples were run on a 5–15% SDS-PAGE and transferred electrophoretically to nitrocellulose. Tyrosine phosphorylation of the receptor was then detected by Western blot with anti-phosphotyrosine antibody (gift of Tom Roberts, Dana Farber Cancer Institute).

In Vivo Kinase Assay—The cells were grown to confluence in 35-mm wells. The medium was exchanged for methionine-free medium with 200 μCi/ml [35S]methionine and incubated for 4 h at 37 °C. The cells were then washed twice with phosphate-buffered saline, twice with isotonic wash, and then overlaid with stimulation buffer (isotonic saline), cells were then washed twice with phosphate-buffered saline, twice with isotonic wash, and then overlaid with stimulation buffer (isotonic saline), and then incubated for 5 min at 37 °C while the cells were still adherent to the culture plates. The buffer was removed, the cells lysed with 800 μl of lysis buffer (as above), and the mixture transferred to 1.5-ml Sarstedt screw-cap tubes for subsequent anti-phosphotyrosine immunoprecipitation.

Anti-phosphotyrosine Immunoprecipitation—Each 800-μl sample was precleared with 100 μl of bovine serum albumin-agarose (Sigma) for 1 h at 4 °C and then incubated with 10 μl of anti-phosphotyrosine antibody linked to agarose beads (Oncogene Science Inc., New York) for 2 h at 4 °C. The resultant bead complexes were washed twice with lysis buffer (as above), twice with a modified lysis buffer (lacking bovine serum albumin), and the product was eluted with 40 μl of elution buffer (lysis buffer with 0.01% ovalbumin substituted for the 0.1% bovine serum albumin and 1.0 mM phenyl phosphate) for 10 min at 4 °C. The beads were removed with microfilterfuge tubes, and the resultant supernatant was subjected to denaturing acrylamide electrophoresis on 5–15% gradient gels (SDS-PAGE).

RESULTS

Morphologic and Growth Characteristics of Normal, Transformed, and Revertant Cells—The characteristic morphology of Balb/c 3T3 fibroblasts at confluence is that of flat appearing cells that demonstrate contact inhibition. Kirsten v-ras-containing fibroblasts (KBalb), in contrast, appear small and refractile with extended processes and overgrow the monolayer at confluence (Fig. 1). Revertants in the transformed phenotype were created by transfection with the Kreu-1 cDNA and selection of clones resistant to G418. The mRNA analysis of these clones (called KR) demonstrated the presence of Kreu-1 transcripts (Fig. 2). Alternatively, the cells were treated with 2 mM dibutyryl cAMP for 48 h. In Fig. 1

![Fig. 1. Morphologic appearance of ras-containing Balb cells and revertants. Panels A, C, and E show photomicrographs of Balb cells, Balb cells treated with cyclic AMP, and Balb cells transfected with the Kreu-1 gene, respectively. Panels B, D, and F show photomicrographs of KBalb cells, KBalb cells treated with cyclic AMP, and KBalb cells transfected with the Kreu-1 gene (KR-1), respectively.](image)

![Fig. 2. Kreu-1 transcripts are expressed in Kreu-1-transfected revertant cell lines. An autoradiogram of a Northern blot of whole cell RNA (30 μg/lane) is shown. RNA was isolated from Balb, KBalb, KR-1, KR-6, and KBalb/cAMP, separated on a denaturing agarose gel, and transferred to nitrocellulose. Hybridization was performed with both the Kreu-1 probe and the control ATPase probe simultaneously. An autoradiogram is shown here. Kreu-1 mRNA was demonstrated in the KR-1 and the KR-6 cells and was not demonstrable in the other cells despite prolonged exposures.](image)

![Table 1. Serum requirements of ras-containing Balb cells and their revertants.](table)

| Cell     | Proliferation assay | 0.5% Serum | 10% Serum |
|----------|---------------------|------------|-----------|
| Balb     | Day 0 control       | Day 4      |           |
|          |                     | 1.0        | 1.7       |
|          | 0.5% Serum          | 49.0       | 42.0      |
| KBalb    | 1.0                 | 49.0       | 42.0      |
| KBalb/cAMP | 1.0                | 2.1        | 14.0      |
| KR-1     | 1.0                 | 3.5        | 35.0      |

the morphology of both of these types of revertants was compared with the parental KBalb cell line. Neither of these revertants approached the appearance of the untransformed Balb cells; however, they were clearly flatter than their parental cell line, KBalb. They also demonstrated contact inhibition. The changes noted in the cAMP-treated cells became apparent after exposure for 24–48 h to 2 mM dibutyryl cAMP. Neither the cAMP treatment nor the Kreu-1 transfection affected the levels of v-ras as detectable by mRNA analysis (not shown). Therefore, the changes noted in the two types of revertants were not caused by the trivial explanation of having reduced the levels of ras in the cells.

The serum requirements of these revertant cell lines were assessed. After growth in 0.5% calf serum or 10% calf serum for a predetermined number of days, the increase in cell number was determined (Table 1). KBalb cells proliferated equally well under either serum concentration, demonstrating the well established decrease in serum requirement which accompanies the transformed state. Proliferation of Balb cells was arrested completely under conditions of 0.5% serum. Both cAMP-treated and Kreu-1 transfected KBalb cells behaved...
DNA Synthetic Response of ras-containing Balb Cells and Their Revertants to PDGF or Serum—The ability of the normal Balb cells, the v-ras transformed cells, and the cAMP-treated or Kreu-1-transfected revertants to respond to PDGF-BB mitogenically was assessed by thymidine incorporation. Tritiated thymidine incorporation over 24 h in Balb cells increased approximately 20-fold in response to 10 ng/ml PDGF-BB (Table II). No such increase in response to PDGF-BB was observed in the KBalb cells (which already possessed a high basal incorporation) although treatment with 10% serum did lead to a small increase. Treatment of KBalb cells with cAMP or the introduction of the Kreu-1 gene produced dramatic results. The basal level of thymidine incorporation during serum deprivation fell, approaching the low level found in the Balb cells (Table II). However, no significant increase in thymidine incorporation was observed in response to PDGF-BB. As a positive control, treatment with 10% serum resulted in a 2-10-fold increase in incorporation for the Kreu-1-transfected cells and the cAMP-treated cells, respectively (compared with a 52-fold increase seen in the Balb cells). This minimal response to serum in the revertants demonstrates that the cells are still capable of responding to growth factors in serum other than PDGF.

Phospholipase C Activity in Response to PDGF-BB—PLC activity in membrane preparations of our cell lines was examined. PIP$_2$ hydrolysis increased 25% in the normal Balb cell membranes upon treatment with PDGF-BB (Fig. 3A). Membranes from v-ras-infected KBalb cells demonstrated a 16% decrease in PLC activity in response to PDGF-BB (Fig. 3A). The differences between the Balb cells and the KBalb cells were highly significant ($p < 0.05$). Membranes from the revertants, as represented by the KBalb/cAMP in Fig. 3A, had an increase of 24% in their PLC activity upon PDGF-BB stimulation, a statistically significant difference ($p < 0.05$) from the decrease seen in the KBalb cells.

We also examined the PLC activity that was recoverable in phosphotyrosine immunoprecipitates of quiescent and stimulated cells. In these preparations, PIP$_2$ hydrolysis increased 28% in the normal Balb cells upon treatment with PDGF-BB (Fig. 3B; $p < 0.05$). Similar to the results obtained using the membrane preparations, immunoprecipitates from the v-ras-infected KBalb cells demonstrated a 44% decrease in PLC activity in response to PDGF-BB (Table II). No such increase in response to PDGF-BB was observed in the KBalb/cAMP cells. The values shown represent the PLC activities demonstrable in the basal state and after stimulation with 40 and 80 ng/ml PDGF-BB. In response to PDGF, PLC activity in the Balb cells increased by 25%; in the KBalb cells, PLC activity decreased by 16%; and in the revertants, PLC activity increased by 24%. These differences were significant ($p < 0.05$).

Panel B, PLC activity was measured as above but in anti-phosphotyrosine immunoprecipitates from Balb, KBalb, KBalb/cAMP, and KR6-5 cells. In response to PDGF, PLC activity in the Balb cells increased by 28%; and in the KBalb cells, PLC activity decreased by 44%. The KBalb/cAMP revertants showed a 17% decrease after PDGF-BB stimulation whereas the KR6-5 revertants showed a 24% increase. This 24% increase in the KR6-5 cells was significant compared with the 44% decrease seen in the KBalb cells ($p < 0.05$).

| Cell          | Basal [cpm] | PDGF [cpm] | Serum [cpm] |
|---------------|------------|------------|-------------|
| Balb          | 11         | 215        | 580         |
| KBalb         | 344        | 380        | 610         |
| KBalb/cAMP    | 9          | 18         | 88          |
| KR6-5         | 48         | 33         | 116         |

**Fig. 3.** Activation of PLC after PDGF stimulation is restored in the revertants. Panel A, PLC activity as represented by cpm of tritiated inositol 1,4,5-trisphosphate (IP3) released after incubation of tritiated PIP$_2$ with quiescent or stimulated membranes isolated from Balb, KBalb, and KBalb/cAMP cells. The values shown represent the PLC activities demonstrable in the basal state and after stimulation with 40 and 80 ng/ml PDGF-BB. In response to PDGF, PLC activity in the Balb cells increased by 25%; in the KBalb cells, PLC activity decreased by 16%; and in the revertants, PLC activity increased by 24%. These differences were significant ($p < 0.05$). Panel B, PLC activity was measured as above but in anti-phosphotyrosine immunoprecipitates from Balb, KBalb, KBalb/cAMP, and KR6-5 cells. In response to PDGF, PLC activity in the Balb cells increased by 28%; and in the KBalb cells, PLC activity decreased by 44%. The KBalb/cAMP revertants showed a 17% decrease after PDGF-BB stimulation whereas the KR6-5 revertants showed a 24% increase. This 24% increase in the KR6-5 cells was significant compared with the 44% decrease seen in the KBalb cells ($p < 0.05$).
there was no significant change seen after treatment with PDGF-BB (data not shown).

**Calcium Mobilization in Response to PDGF-BB**—A technique for examining stimulus-dependent calcium mobilization in fibroblast cells in suspension was developed to permit analysis of the response of an entire population of cells rather than relying on the pooling of single cell analyses. Basal fluorescence of the INDO-1-loaded cells was identical for each cell type, indicating that intracellular pools of calcium did not differ in the presence of the v-ras or Kreu-1 genes. The presence of v-ras markedly inhibited the mobilization of calcium which is normally observed in Balb cells in response to PDGF-BB, decreasing it by 80–92% (Fig. 4). Transfection of KBalb cells with Kre-1 resulted in substantial restoration of the intracellular calcium response to PDGF-BB, to 80–83% of the Balb levels of calcium mobilization (Fig. 4). Both the characteristic 5-s delay between the addition of PDGF-BB and the beginning of the calcium flux and the distinctive shape of the INDO-1 profile were restored in the Kre-1-containing cells (tracings not shown). Cyclic AMP-treated KBalb cells were not evaluable by the method used, as the pretreatment with CAMP inhibited loading with the indicator dye.

**PDGF-BB-mediated Induction of Growth-related Genes**—We have shown previously that in v-ras-containing cells, PDGF-BB stimulated induction of the growth-related genes c-myc, c-fos, and JE was inhibited. This inhibition was at the level of transcription and was apparent within 30 min of v-ras introduction into the cell (Zullo and Faller, 1988). Although inducible by PDGF-BB, c-myc mRNA expression appeared to become somewhat deregulated when Balb cells transformed by v-ras were carried over many generations, such that basal steady-state levels of c-myc mRNA are higher in serum-deprived v-ras-containing cells than in the untransformed parental cell line.

The revertants were subjected to mRNA analysis to examine the transcript levels of these genes, before and after PDGF-BB stimulation, in comparison with the parental lines. Balb cells showed a strong induction of c-fos transcripts by 30 min after treatment with PDGF-BB, whereas c-fos transcripts in the PDGF-treated KBalb cells were only detectable on a longer exposure and were at least 100-fold less than the untransformed cells by quantitation using densitometric scanning (Fig. 5, top). The revertant cell lines (KR-1 or KBalb/CAMP) demonstrated strong induction of c-fos mRNA in response to PDGF-BB, with the level of mRNA induction approaching that of normal Balb cells (Fig. 5, top). In the Kreu-1-containing cells (KR-1), the level of induction of c-fos mRNA was dependent on the cell density; that is, c-fos transcripts in more confluent cultures were somewhat less inducible (not shown). The CAMP-treated cells demonstrated full induction of c-fos regardless of cell density.

The KBalb cells had an undetectable base-line JE mRNA level and a very low level of induction after PDGF-BB stimulation when compared with the Balb cells (Fig. 5, bottom). In both the Kreu-1-containing revertants and in the CAMP-treated KBalb cells, there was a strong increase in the level of JE mRNA seen after PDGF-BB stimulation, but there was also a rise in the basal (unstimulated) level.

Some constitutive expression of c-myc mRNA was observed in the serum-deprived KBalb cells, but this expression was unchanged in response to PDGF-BB (Fig. 5, bottom). The Kreu-1-containing revertants (KR-1 or KR-6) and the CAMP-treated revertants demonstrated two significant changes in comparison with the KBalb cells: 1) reduction of the basal levels of transcripts for c-myc in serum-deprived cells; and 2) restoration of PDGF-BB inducibility. The KR- and CAMP-treated cell lines had c-myc mRNA levels that were undetectable.
able in the quiescent unstimulated state. In response to PDGF-BB, c-myc transcripts were induced to levels comparable to those found in the PDGF-BB-stimulated Balb cells (Fig. 5, bottom).

Exogenous PLC or PMA Induces c-myc Transcripts in ras-containing Balb Cells—Calcium immobilization and induction of the growth-related gene c-myc by PDGF are generally considered to be events dependent upon activation of PLC. Since these two events are also inhibited in v-ras-containing cells the possibility existed that the inhibition of PDGF-BB signal transduction in the KBalb cells was at the level of PLC activation. To test this possibility serum-starved KBalb cells were treated directly with PLC or with PMA, a known activator of protein kinase C.

The results of RNA blot analysis showed that both PLC and PMA were able to increase the mRNA levels of the c-myc transcripts in the KBalb cells (Fig. 6). These results suggest that the block to PDGF-BB signal transduction in the v-ras-containing cells is at or before the level of PLC activation and the protein kinase C-mediated components of the signal cascade. We have shown previously that there is a blockade at the level of receptor phosphorylation in v-ras-containing cells (Rake et al., 1991). This new result demonstrates that the signaling pathway distal to the activation of PLC is still intact in the KBalb cells.

PDGF Receptor Autophosphorylation Remains Blocked—Balb cells demonstrate autophosphorylation of the PDGF receptor when stimulated by PDGF-BB. When Balb fibroblasts were labeled metabolically with [35S]methionine and the cytoplasmic membranes solubilized in 0.1% Triton X-100, the autophosphorylated receptor could be immunoprecipitated with an anti-phosphotyrosine antibody and identified as the band migrating at 180 kDa (Fig. 7, top). Under these same conditions autophosphorylation of the PDGF receptor in KBalb cells was not demonstrable (Rake et al., 1991). Furthermore, the revertants obtained after exposure of KBalb fibroblasts to 2 mM dibutyryl cAMP for 48 h did not tyrosine phosphorylate the PDGF receptor after stimulation with PDGF-BB (Fig. 7, top). The Krev-1-containing revertants (KR) demonstrated the same inhibition to tyrosine phosphorylation of the PDGF receptor in [35S]methionine-labeled cells (not shown), and this was confirmed further in the Krev-1-containing revertants utilizing different assay conditions: digitonin permeabilization, labeling with [γ-32P]ATP, and immunoprecipitation with the same anti-phosphotyrosine antibody (Fig. 7, bottom).

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Fig. 6. Exogenous PLC or PMA induces c-myc transcripts in ras-containing Balb cells. Confluent Balb and ras-containing Balb (KBalb) cells were serum starved (0.5% serum) for 24 h and then treated with either 2 units/ml purified B. cereus PLC, 20 ng/ml PMA, 10% calf serum (CS), or no addition (none) for 90 min, at which time the cells were lysed with guanidine thiocyanate and their RNA purified. The total cellular RNA (20 μg/lane) was size fractionated on a formaldehyde-agarose gel, transferred to a nitrocellulose filter, and the filter hybridized simultaneously with radiolabeled probes for c-myc and ATPase (as a control). The addition of 10% calf serum was used as a positive control for induction of c-myc levels in KBalb. An autoradiogram is shown here. There were significant increases in the levels of c-myc transcripts in both the Balb and KBalb cells in response to both PLC and PMA.
tyrosine phosphorylation of protein bands at 145, 100, 85, 74, and 42 kDa (Fig. 9). No unequivocal identification of these other proteins seen in Figs. 8 and 9 has been established, but the molecular masses of the 145-, 85-, 74-, 60-, and 42-kDa bands suggest that they may be the previously identified substrates of membrane-associated receptor tyrosine kinase activity: PLC, PI-3 kinase, Raf-1, src, and pp42, respectively (Meisenhelder et al., 1989; Kaplan et al., 1987; Morrison et al., 1989; Kypert et al., 1990; Rossomando et al., 1989; Williams, 1989). Phosphorylation of these presumptive receptor kinase substrates in response to PDGF-BB was never observed in the KBalb cells and was not restored in the cAMP or in the Kreu-1 revertant cells. Of note, the inhibition of tyrosine phosphorylation of the 100-kDa protein in response to PDGF was an inconsistent finding in that occasional experiments did demonstrate PDGF-BB-stimulated phosphorylation of the 100-kDa protein in the KBalb cells using the in vivo method. None of the other PDGF receptor substrate proteins found in the KBalb cells or in the revertants demonstrated any significant tyrosine phosphorylation under either of the conditions shown in Figs. 8 and 9.

DISCUSSION

The presence of activated ras proteins in the cell blocks the PDGF-BB-mediated activation of the suspected second messenger components of the signal transduction pathway, including PDGF receptor autophosphorylation, phosphorylation of the PDGF receptor-associated substrates, activation of phospholipase C, calcium mobilization, and induction of the growth-related genes. These potential second messengers were initially thought to be “linked,” consistent with a linear model of signal transduction in which one event activates the next. If this model were correct then inhibition of any point in this signaling cascade by v-ras would block activation of all of the subsequent components of the pathway; accordingly, reversion of this blockade would result in reconstitution of all the second messenger elements. Alternatively, if more than one distinct and independent signal are generated by the binding of PDGF-BB to its receptor (a branching model for the signal transduction cascade) then revertants of ras transformed cells might demonstrate reconstitution of certain signaling components while others would remain blocked. Increasing indirect evidence has been accumulating which suggests that the presumed second messengers of PDGF signaling may be acting independently of one another, consistent with a branching model for signal transduction (Williams, 1989). A comparison of the v-ras-containing cells we describe here with the morphological revertants produced by cAMP or Kreu-1 provides further evidence for a branching model by demonstrating uncoupling among some of the second messengers and accordingly aids in the assignment of specific second messengers to one or another arm of such a model.

The mechanisms by which Kreu-1 or cAMP induce morphologic reversion have not yet been elucidated. The Kreu-1 gene encodes a 21-kDa ras-like protein that is identical to the previously reported smg21 and rap1 and which reverts v-ras transformed cells to a more normal morphology (Kitayama et al., 1989; Pizon et al., 1988). Cyclic AMP causes a similar reversion (Carchman et al., 1974; Anderson et al., 1974) and has been documented to reverse at least partially the inhibition by v-ras of PDGF-BB-induced calcium mobilization and PI turnover (Olinger et al., 1989). The Kreu-1 transfectants and the cAMP-treated KBalb cells we describe have growth characteristics and serum requirements that approach those of the normal Balb fibroblasts; yet their mitogenic response
with phorbol esters or exogenous phospholipase C results in fos,
more, we have shown that treatment of Balb or KBalb cells
results in activation of PLC and growth-related gene transcription, it was
measured by thymidine incorporation, is clearly not restored, and it is evident that only certain of the
PDGF second messenger signals which were blocked by v-ras have been reconstituted in the revertants.

The PDGF-BB-mediated induction of c-myc, c-fos, and JE transcripts was restored substantially in the revertant cells. One point is noteworthy regarding the c-myc mRNA levels. In the revertants there was not only a restoration of the induction of c-myc mRNA in response to PDGF-BB, but there was also a reversal of the deregulation of c-myc mRNA levels which is characteristic of chronically v-ras-infected cells. Deregulation of c-myc expression resulting in high constitutive c-myc mRNA levels is a phenotype common to many transformed cell lines and naturally occurring tumors and is thought to contribute to tumorigenesis (Bading and Moelling, 1990; Forgue Lafitte et al., 1989; Zajac-Kaye and Levens, 1990; Kakkis et al., 1989; Suchy et al., 1989). In our hands, Balb cells containing activated ras genes also have this phenotype. It may be significant that the morphological revertants also demonstrate reversal of this deregulated phenotype.

The mechanism for PDGF-mediated induction of c-myc, c-fos, and JE transcripts may be independent of the PDGF receptor kinase activity, or at least of receptor autophosphorylation, since in neither type of revertant was there significant ligand-induced tyrosine phosphorylation of the PDGF receptor; nor was there a restoration of a PDGF-mediated increase in phosphatidylinositol-3 kinase activity in either revertant. This kinase-independent restoration of growth-related gene induction may be mediated by PLC, as the ability of PDGF to induce both growth-related gene transcription and PLC activation reverted coincidentally in the Krev-1- and CAMP-treated cells. The revertant cells demonstrate a clear restoration of PDGF-inducible PLC activity by direct measurement of PIP₂ hydrolysis, and by calcium mobilization, which is presumed to be a PLC-dependent phenomenon. PDGF-inducible PLC activity was restored in these cells without any detectable ligand-stimulated receptor tyrosine kinase activity.

In view of current speculation regarding the role of receptor tyrosine kinase activity in growth factor signaling and our finding of a restoration of PDGF-mediated induction of both PLC activity and growth-related gene transcription, it was striking that the kinase activity of the PDGF receptor was not restored in the Krev-1 or dibutyryl CAMP-treated revertants. The mechanism of the delivery of a PDGF-mediated signal resulting in the induction of c-myc, c-fos, and JE in the absence of phosphorylation of the receptor remains undefined. There may be a conformational change in the PDGF receptor which is sufficient to activate some other second messenger pathway distinct from the PDGF receptor tyrosine kinase-dependent pathway and which results in the activation of the growth-related genes. This tyrosine kinase-independent pathway would then potentially represent a branch point in signal transduction for PDGF. Both the kinase-independent and kinase-dependent arms of the pathway appear to be necessary for the full delivery of the proliferative signal to the cell, yet each arm could be dissociated from the other one under conditions like those described here.

We propose that the PDGF-receptor kinase-independent arm of the signaling pathway might be mediated by activation of phospholipase C. We have demonstrated substantial restoration of PIP₂ hydrolysis and calcium mobilization in response to PDGF-BB in the Krev-1- or CAMP-induced revertants, demonstrating reactivation of PLC activity. Furthermore, we have shown that treatment of Balb or KBalb cells with phorbol esters or exogenous phospholipase C results in a pattern of growth-related gene expression identical to that seen in PDGF-treated Balb cells, which would indicate that events distal to the activation of PLC are not directly inhibited in the v-ras-containing cells. It is possible that the PDGF receptor transmits a signal to phospholipase C via a conformational change that is sufficient to lead to the induction of the genes c-myc, c-fos, and JE (and c-jun²). Our in vivo kinase assays did not demonstrate increased tyrosine phosphorylation of the 145-kDa protein. When the PLC activities in the phosphotyrosine fractions of soluble cell lysate were examined no consistent pattern for PLC activity could be demonstrated among the revertants despite a consistent restoration of PLC activity in both types of revertants. These findings suggest that changes in tyrosine phosphorylation of PLC may not be the mechanism for its activation in these particular revertants. However, until the phosphorylated state of receptor-associated and unassociated PLC in the revertants is examined more directly phosphorylation of PLC by the receptor or by other activatable kinases in the revertants cannot be ruled out. Nonetheless, it is likely that the tyrosine kinase activity of the PDGF receptor itself is necessary for the initiation of certain other signaling pathways in the cell which together with the non-kinase-dependent signals are required for DNA synthesis and cell growth.

It is noteworthy that induction of growth-related gene expression, including c-myc, by PDGF-BB in the revertants is not sufficient for stimulation of DNA synthesis. This is unexpected in light of previous reports that microinjection of c-myc protein is sufficient to move cells into S phase of the cell cycle (Kaczmarek et al., 1988). There are a number of trivial explanations for these divergent findings, including dose effects, e.g. the amount of c-myc protein delivered by microinjection is supraphysiologic whereas the induction of c-myc mRNA we noted in the revertant cells is likely to be of the same magnitude as that seen in the normal Balb cells. Yet, our findings indicate the likely presence of at least two signaling pathways induced by ligand binding to the PDGF receptor which remain distinct beyond the induction of the immediate early genes and which together must be activated for effective growth factor-mediated DNA synthesis.

In summary, we have utilized revertants of v-ras-containing (PDGF-unresponsive) fibroblasts induced by either treatment with CAMP or transfection with Krev-1 to study the PDGF signaling pathway. These cells did not revert the block in autophosphorylation and presumed kinase activity of the PDGF receptor but have demonstrated restoration of the PDGF-BB-mediated increase in PLC activity and induction of c-myc, c-fos, and JE. This provides evidence that autophosphorylation of the intact PDGF receptor can be dissociated from PLC activation and the induction of the growth-related genes. These findings may suggest the presence of a PDGF receptor tyrosine kinase-independent portion of the PDGF signal transduction pathway, a portion that needs to be coupled with the kinase-dependent portion for the full delivery of the signal into the cell.

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² M. A. Quiñones, L. J. Mundschau, J. R. Rake, and D. V. Faller, unpublished observations.
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