

# Protein Kinase C Mediates Platelet-derived Growth Factor-induced Tyrosine Phosphorylation of p42

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**Abstract.** One of the early events after stimulation of Swiss 3T3 cells with either platelet-derived growth factor (PDGF), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), diacylglycerol, or several other mitogens is the near stoichiometric phosphorylation at tyrosine and serine of a scarce cytoplasmic protein (p42). TPA and diacylglycerol are known to directly stimulate the activity of a protein-serine/threonine kinase, protein kinase C (PKC). PDGF and several other mitogens stimulate tyrosine kinases directly and PKC indirectly. We have therefore examined the involvement of PKC in p42 tyrosine phosphorylation in Swiss 3T3 cells. Firstly, six agents which stimulated phosphorylation of p42 also stimulated phosphorylation of a known PKC substrate, an 80,000-*M<sub>r</sub>* protein (p80). Secondly, in PKC-deficient cells (cells in which PKC activity was

reduced to undetectable levels by prolonged exposure to TPA), PDGF-induced p42 phosphorylation was reduced three- to fourfold. Phosphoamino acid analysis of phosphorylated p42 from PDGF-stimulated PKC-deficient cells revealed primarily phosphoserine and only a trace of phosphotyrosine, suggesting that the reduction in PDGF-stimulated tyrosine phosphorylation of p42 resulting from PKC deficiency is greater than three- to fourfold. Finally, comparison of anti-phosphotyrosine immunoprecipitates of PKC-deficient versus naive cells revealed that most other PDGF-induced tyrosine phosphorylation events were quite similar. These data suggest that mitogens such as PDGF, which directly stimulate phosphorylation of some proteins at tyrosine, induce p42 tyrosine phosphorylation via a cascade of events involving PKC.

**T**HE interaction between mitogenic agents and their target cells usually proceeds via specific cell surface receptors, which in numerous instances are protein-tyrosine kinases (24, 27). In these cases, mitogen binding stimulates protein phosphorylation at tyrosine, and this, or other as yet unknown activities of the receptor, initiates multiple changes within the cell. One of these changes is the accelerated turnover of phosphoinositides, which leads to increases in intracellular concentrations of both  $Ca^{2+}$  and membrane diacylglycerol (DAG)<sup>1</sup> (2). This triggers protein phosphorylation at serine and threonine by protein kinase C (PKC), a family of  $Ca^{2+}$ /phospholipid-dependent protein kinases (26, 31, 34, 44). Direct activation of PKC by phorbol diesters, or indirect activation by mitogens such as thrombin, platelet-derived growth factor (PDGF), and bombesin, stimulates the proliferation of many cell types (7, 25, 28, 34, 36, 39, 40), suggesting that PKC activation via increased phosphoinositide turnover is an important part of the response to many mitogens.

Among the proteins identified to be phosphorylated at tyrosine in response to mitogenic stimulation is p42—an evolutionarily conserved cytoplasmic protein constituting

~0.002% of total protein (4, 12, 16, 21, 32, 33). Within 10 min of adding any of several different mitogenic agents, ~50% of p42 molecules become phosphorylated on tyrosine and serine (12). Quantitatively, phosphorylated p42 (pp42A and pp42B) and related phosphoproteins (pp45A and pp45B) contain a large proportion of the phosphotyrosine in a stimulated cell, as detected by one-dimensional (33) or two-dimensional electrophoresis of total phosphoproteins (12).

The kinase which phosphorylates p42 on tyrosine is unknown. Induction of tyrosine phosphorylation of p42 by those mitogens that activate receptor protein-tyrosine kinases could result from the direct action of receptor tyrosine kinases. Alternatively, activation of the receptor kinase could indirectly stimulate one or more other protein-tyrosine kinases that phosphorylate p42. It is also possible that the phosphotyrosine content of p42 could be increased if the activity of a specific phosphatase were inhibited, or if the conformation or localization of p42 were modified so that its interactions with a constitutively active kinase or phosphatase were altered. The possibility of both serine and tyrosine kinases being involved in p42 tyrosine phosphorylation is suggested by the observation that compounds such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and DAG stimulate p42 tyrosine phosphorylation even though they appear not to stimulate protein-tyrosine kinases directly (4, 16, 21, 32). Indeed, the data presented here suggest that there exists a

1. *Abbreviations used in this paper:* DAG, diacylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C.

PKC-regulated p42-specific enzyme responsible for mitogen-induced phosphorylation of p42.

## Materials and Methods

### Materials

PDGF, purified by a modification of the Raines and Ross protocol (38), was a generous gift of Paul Dicorleto of the Cleveland Clinic Foundation, Cleveland, OH. Monoclonal antibody against phosphotyrosine (IG2) coupled to Sepharose 4B was generously provided by Raymond Frackelton of Brown University, Providence, RI. Epidermal growth factor (EGF) was obtained from Collaborative Research, Lexington, MA; TPA, DAG (1-oleoyl-2-acetyl-*rac*-glycerol), cAMP-dependent protein kinase inhibitor, and histone HI (III S) were from Sigma Chemical Co., St. Louis, MO.

### Cell Culture

Murine Swiss 3T3 cells were maintained in DME supplemented with 5% calf serum.

### Metabolic Labeling and Lysis of Cells

Confluent, quiescent cultures of cells were washed two times in H/S buffer (20 mM Hepes, pH 7.4, 150 mM NaCl) and incubated overnight in labeling medium (DME containing 10% of the normal phosphate concentration, 15 mM Hepes, pH 7.4, 0.1% calf serum).  $H_3^{32}PO_4$  was added to a final concentration of 1 mCi/ml (unless indicated otherwise) and the incubation at 37°C continued for 4 h. When labeling with [ $^{35}S$ ]methionine, the same protocol was followed, except that the labeling medium lacked methionine and had the normal concentration of phosphate. After an overnight incubation in the methionine labeling medium, [ $^{35}S$ ]methionine was added to a final concentration of 0.2 mCi/ml and incubated for an additional 4 h. The desired mitogens were added during the last 10 min of the 4-h incubation, after which cells were transferred to ice and washed twice with ice-cold H/S buffer.  $2 \times 10^5$  cells were lysed in 100  $\mu$ l of lysis buffer (0.3% SDS, 65 mM dithiothreitol [DTT], 1 mM EDTA, 20 mM Tris, pH 8.0), which was preheated to 100°C immediately before addition to cells. The dishes were scraped with a rubber policeman, and 0.1 vol of DNase-RNase (20) was added. The sample was transferred to a microfuge tube, vortexed, incubated 1 min at 0°C, frozen on dry ice, lyophilized, resuspended in sample buffer (20), and stored at -70°C.

Samples prepared by this procedure from mitogen-stimulated cells show increased tyrosine phosphorylation of pp42A and pp45A, with only slight phosphorylation of related proteins pp42B and pp45B. A different sample preparation procedure (20) yields samples in which pp42B and pp45B are more highly labeled than pp42A and pp45A (13, 16, 21). We do not know which procedure more accurately represents the situation in the intact cell, but both procedures are highly reproducible.

### Two-dimensional Electrophoresis

Samples were separated in the first dimension by IEF, using pH 3.5-10 ampholytes (unless otherwise indicated), followed by SDS-PAGE (15% acrylamide, 0.375% bis acrylamide) in the second dimension, as previously described (10, 20). Acidic proteins focused to the left of the gels. Samples were normalized on the basis of cell number. Unless otherwise stated,  $2 \times 10^4$  cells were loaded per gel. For alkali treatment (8, 10), dried gels were rehydrated, incubated in 1 M KOH for 2 h at 56°C, fixed in 10% acetic acid and 10-20% isopropanol, and dried under vacuum. All gels were exposed with intensifying screens at -70°C.

### Phosphoamino Acid Analysis

The phosphoamino acid content was determined as previously described (15). Briefly, gels were washed in water and dried after electrophoresis; proteins were located by autoradiography, extracted, TCA precipitated, and partially hydrolyzed by incubating in 5.7 N HCl for 1 h at 110°C. The resulting phosphoamino acids were applied onto a 100- $\mu$ m cellulose plate and separated by two-dimensional thin layer electrophoresis, first at pH 1.9, followed by electrophoresis at pH 3.5 in the second dimension. Authentic phosphoamino acids, mixed into the sample, were visualized by staining with ninhydrin (0.25% in acetone).

### Immunoprecipitation

Confluent, quiescent, 90-mm dishes of Swiss 3T3 were metabolically labeled with  $H_3^{32}PO_4$  and stimulated with the desired mitogen as described above. Dishes of cells were placed on ice and quickly cooled by washing twice with H/S buffer. Cells were lysed and immunoprecipitated as described by Frackelton et al. (18). Briefly, 0.4 ml of ice-cold EB (1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 2 mM  $Na_3VO_4$ , 0.1% BSA, 1% Aprotinin, 10 mM Tris, pH 7.6, 1 mM phenylmethylsulfonyl fluoride [PMSF]) was added, and the cells were scraped with a rubber policeman and transferred to a microfuge tube on ice. The culture dish was rinsed with an additional 0.4 ml of EB which was combined with the first extraction. Samples were incubated on ice with occasional vigorous vortexing for 20 min, and centrifuged at 15,000 g for 15 min at 4°C to remove insoluble debris. The supernatant was incubated with 100  $\mu$ l of Sepharose CL-4B beads for 30 min at 4°C with continual mixing. The precleared samples were added to fresh tubes containing 10  $\mu$ l Sepharose 4B to which the monoclonal antibody to phosphotyrosine (IG2) had been covalently attached, and incubated for 2 h at 4°C with inversion mixing. The beads were washed three times with EB, twice with EB without BSA, and the antigen was eluted by incubating for 10 min on ice with gentle vortexing in eluting buffer (EB with 1 mM phenyl phosphate, 0.01% ovalbumin instead of 0.1% of BSA, and 10 mM NaCl instead of 50 mM). Eluted samples, normalized on the basis of cell number, were analyzed by two-dimensional electrophoresis as described above.

### Down Regulation

Confluent, quiescent cultures of cells were incubated at 37°C in DME, 1% calf serum containing 1  $\mu$ g/ml TPA for ~30 h, washed twice with H/S buffer, and incubated at 37°C overnight in labeling medium containing 1  $\mu$ g/ml TPA.

### Histone Phosphorylation

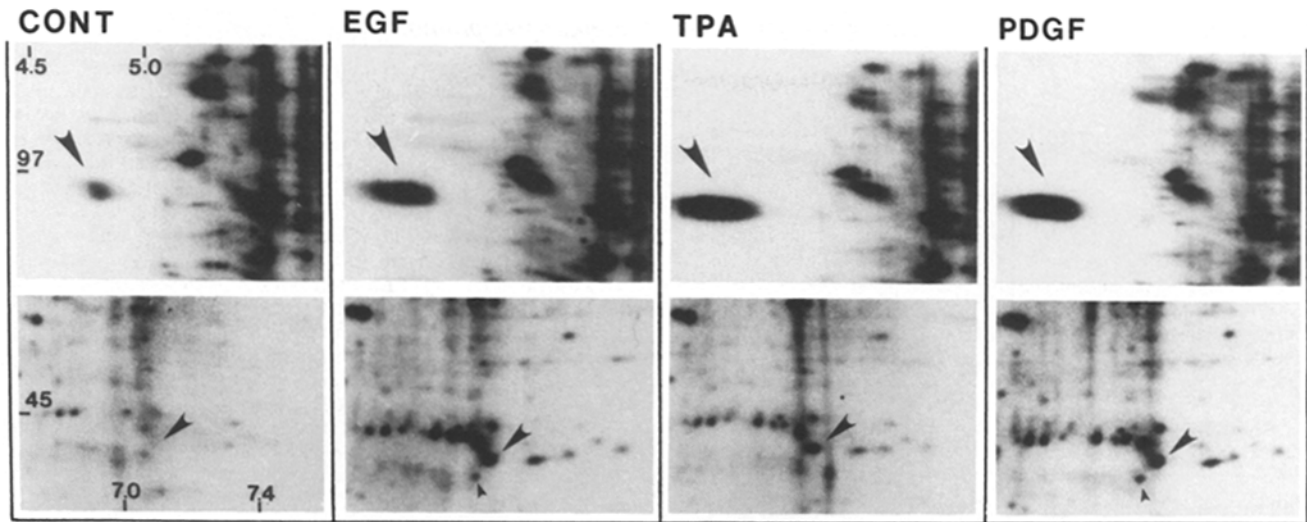
PKC activity was quantitated in crude cell lysates essentially as described by Pelech et al. (35). 35-mm dishes of cell were rinsed twice with ice-cold H/S buffer and solubilized in 0.4 ml buffer A (35) + 0.5% Triton X-100. 10  $\mu$ l of these extracts was incubated with or without 8  $\mu$ g/ml trypsin for 10 min at 30°C. Excess aprotinin was added to all tubes; and then  $MgCl_2$ ,  $^{32}P$ -ATP, cAMP-dependent protein kinase inhibitor, and histones HI were added to a final concentration of 15 mM, 10  $\mu$ M, 1  $\mu$ M, and 1 mg/ml, respectively, and incubated for 15 min at 30°C. Aliquots were spotted onto P-81 phosphocellulose paper, which was washed five times in 140 mM phosphoric acid and the radioactivity was quantitated by Cerenkov counting.

## Results

### Phosphorylation of p42 and of p80

Although the PDGF receptor kinase is activated after PDGF stimulation, it may not directly phosphorylate p42. Other mitogens able to induce tyrosine phosphorylation of p42 include TPA and DAG, neither of which stimulates the PDGF receptor kinase. However, PDGF, TPA, and DAG are similar in their ability to activate PKC (23, 34). Thus, we tested the theory that PKC may be involved in mitogen-stimulated tyrosine phosphorylation of p42.

If tyrosine phosphorylation of p42 is dependent on PKC activity, then agents that stimulate p42 tyrosine phosphorylation should also activate PKC. As a measure of PKC activity we examined the phosphorylation of p80, which is a well-characterized *in vivo* and *in vitro* PKC substrate (1, 5, 6, 28, 41, 46). As shown in Fig. 1, EGF, TPA and PDGF stimulated both p80 phosphorylation (large arrowhead) and p42 phosphorylation (small arrowhead). In addition, DAG (200  $\mu$ g/ml), calf serum (10%), and even precipitates of calcium pyrophosphate (reported to stimulate DNA synthesis [reference 42]), stimulated both p80 and p42 phosphorylation



**Figure 1.** Phosphorylation of p80 and p42.  $^{32}\text{P}$ -labeled Swiss 3T3 cells were incubated for 10 min at  $37^\circ\text{C}$  with no additions (*CONT*), EGF (100 ng/ml), TPA (100 ng/ml), or PDGF (10 ng/ml). Samples containing equal numbers of cells were analyzed by two-dimensional electrophoresis as previously described (10, 20). The values along the top and bottom indicate pH of the IEF gels, while the molecular mass in kilodaltons is indicated along the left side. Each panel of this figure is a composite of two smaller panels; the upper panel is a portion of a nonalkali-treated gel containing pp80 (*large arrowhead*), while the lower panel is a portion of an alkali-treated gel containing pp42A (*small arrowhead*). The tiny arrowhead in the lower quadrant of the EGF and PDGF panels points to pp41.

(data not shown). Furthermore, insulin (which does not stimulate PKC activity [reference 29]) did not induce phosphorylation of either p80 or p42 (data not shown). Thus p42 is phosphorylated when PKC is activated.

#### **p42 Phosphorylation in PKC-deficient Cells**

Exposure of cultured cells to TPA activates PKC, presumably by mimicking the action of DAG, the physiological activator of this enzyme (34). However, TPA is stable relative to DAG, resulting in prolonged activation of PKC. Because the degradation of PKC is enhanced when PKC is activated, but its synthesis rate remains unchanged, exposure of cells to TPA for extended periods of time results in PKC-deficient cells (6, 9, 41, 44).

Cells were exposed to 1  $\mu\text{g/ml}$  TPA for 48 h (see Materials and Methods), and the PKC activity was quantitated according to the method of Pelech et al. (35). As shown in Table I, the PKC activity was reduced to undetectable levels by prolonged exposure to TPA (the minimum level of detection is  $\sim 15\%$  as estimated from the average of the ranges). We called such cells "PKC-deficient," and control cells "naive."

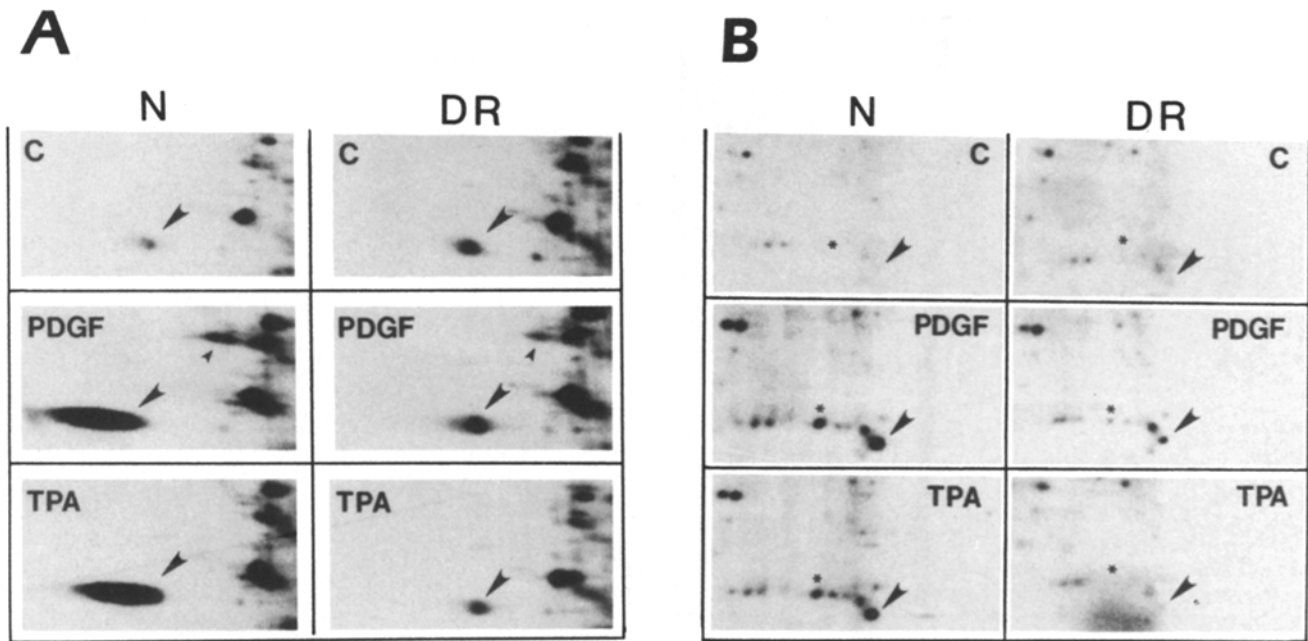
**Table I.** Trypsin-sensitive Histone H1 Phosphorylation

Cells	-Trypsin	+Trypsin	Trypsin stimulation
Naive cells	12,500 $\pm$ 2,400	36,500 $\pm$ 1,100	24,000 $\pm$ 3,500
PKC deficient cells	11,500 $\pm$ 1,200	11,300 $\pm$ 2,900	-200 $\pm$ 4,100

PKC activity was measured in crude cell lysates of naive and PKC-deficient Swiss 3T3 cells by examining trypsin-inducible histone phosphorylation according to the method of Pelech et al. (35). Similar results were obtained in replicate independent experiments. Values displayed are cpm incorporated, mean  $\pm$  range of duplicates.

As reported by others (6, 17, 41), induction of p80 phosphorylation by either TPA or PDGF was dramatically reduced in PKC-deficient cells, although the unstimulated level of p80 phosphorylation was increased relative to naive cells (Fig. 2 A, large arrowhead). Using such PKC-deficient Swiss 3T3 cells, we tested whether the activation of PKC was mandatory for tyrosine phosphorylation of p42. Both the PDGF- and TPA-induced phosphorylation of pp42A (*small arrowhead*) and pp45A (*asterisk*) was markedly reduced in PKC-deficient cells relative to naive cells (Fig. 2 B). Quantitating pp80 (from nonalkali-treated gels) and pp42 (from alkali-treated gels) densitometrically revealed that in PKC-deficient cells, the PDGF- and TPA-induced phosphorylation of p80 was reduced 5.0- and 10.4-fold, respectively, while p42 phosphorylation was reduced 4.9- and 7.0-fold in response to PDGF and TPA, respectively (Table II). Quantitation of pp42A excised from replicate nonalkali-treated gels by Cerenkov counting revealed that PDGF-induced phosphorylation of p42 was reduced 3.5-fold in PKC-deficient cells. Since the reduction of p42 phosphorylation in PDGF-stimulated cells is most apparent at tyrosine (see below), and because the ratio of phosphotyrosine to phosphoserine is increased in alkali-treated gels, the 4.9-fold reduction of p42 phosphorylation due to PKC deficiency, measured from alkali-treated gels, probably more accurately reflects the extent of attenuation of p42 tyrosine phosphorylation. Phosphorylation of p80 and p42 in response to calf serum (10% concentration) or EGF (100 ng/ml) was also greatly reduced in PKC-deficient cells (not shown). These results have been consistently obtained in numerous experiments in Swiss 3T3 cells, as well as with two other cell types (NR-6 3T3 [reference 37] and B-82, a line of mouse L-cells).

Because pp42 contains both phosphoserine and phosphotyrosine (13), it was important to determine the phosphoamino acid composition of pp42 in PDGF-treated PKC-



**Figure 2.** PDGF- and TPA-induced phosphorylation of p80 and p42 in naive vs. PKC-deficient cells. Naive (*N*) or PKC-deficient (*DR*) Swiss 3T3 cells were metabolically labeled with  $H_3^{32}PO_4$ , incubated with no additions (*C*), TPA (100 ng/ml), or PDGF (10 ng/ml) for 10 min at 37°C, lysed, and analyzed by two-dimensional electrophoresis as in Fig. 1. (*A*) Portions of autoradiograms of nonalkali-treated gels illustrating pp80 (large arrowhead) and PDGF receptor (small arrowhead) (19). (*B*) Portions of autoradiograms of alkali-treated gels from naive and PKC-deficient cells containing pp42A (arrowhead) and pp45A (asterisk).

deficient cells. Phosphoamino acid analysis of pp42A from PDGF-treated naive cells exhibits more phosphate on tyrosine than serine (Fig. 3 *A*), whereas pp42A from PDGF-treated PKC-deficient cells contains primarily phosphoserine and only a small amount of phosphotyrosine (Fig. 3 *B*). Thus down regulation of PKC reduced PDGF-induced phosphorylation of p42 at tyrosine, and slightly stimulated p42 serine phosphorylation. These data suggest that the presence of functionally active PKC is required for the phosphorylation of p42 on tyrosine.

#### Abundance of p42 in Naive and PKC-deficient Cells

The reduction of PDGF-induced phosphorylation of p42 in PKC-deficient cells could simply represent a smaller pool of p42 (the unphosphorylated form of pp42A). To deter-

mine whether the process of down regulation of PKC alters the abundance of p42, we compared the amount of p42 in [ $^{35}S$ ]methionine-labeled PKC-deficient vs. naive Swiss 3T3 cells. It is evident from Fig. 4 that the amount of p42 (medium-sized arrowheads pointing left) in naive (Fig. 4 *A*) and PKC-deficient (Fig. 4 *B*) cells is quite similar. Stimulation of naive cells with PDGF diminished the abundance of p42 (Fig. 4 *C*, medium-sized arrowhead pointing left) concomitant with the appearance of pp42A (Fig. 4 *C*, large arrowhead). In PKC-deficient cells, however, stimulation with PDGF resulted in the appearance of very little pp42A (Fig. 4 *D*, large arrowhead), and the abundance of p42 relative to other cell proteins (e.g., small arrowhead pointing right) was not markedly affected. Thus, while both cell types have com-

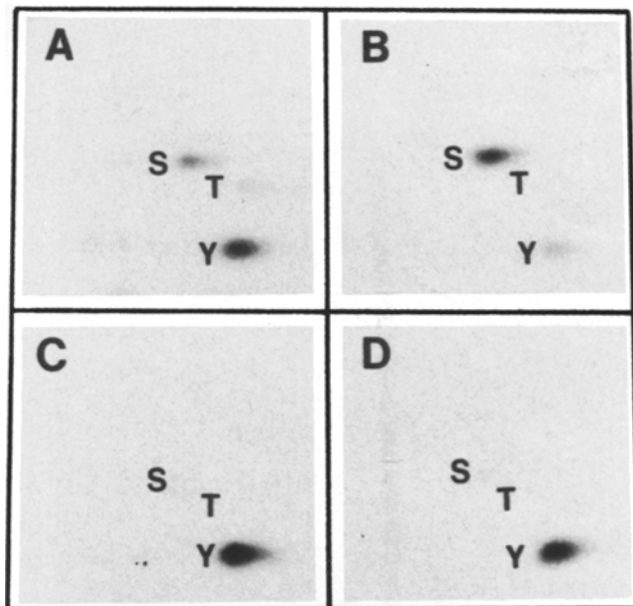
**Table II.** Quantitation of pp80 and pp42 from Naive and PKC-deficient Cells

	Integrated intensity		Stimulated/control*		Naive/PKC deficient‡
	Naive	PKC deficient	Naive	PKC deficient	
pp80					
C	2.3	6.3	1.0	1.0	0.4
PDGF	61.6	12.4	26.8	2.0	5.0
TPA	57.1	5.5	24.8	0.9	10.4
pp42					
C	0.1	0.4	1.0	1.0	0.3
PDGF	12.3	2.5	82.5	5.6	4.9
TPA	4.2	0.6	28.2	1.3	7.0

pp80 and pp42 were quantitated densitometrically with a Visage system equipped with a Eikonix camera from Bio Image, Ann Arbor, MI. pp80 was quantitated from autoradiograms of untreated gels exposed for 1 d, and pp42 from autoradiograms of alkali-treated gels exposed for 4 d.

\* Calculated by dividing the integrated intensity of PDGF- or TPA-stimulated samples by the integrated intensity of control samples.

‡ Calculated by dividing the integrated intensity value of naive by PKC-deficient samples.

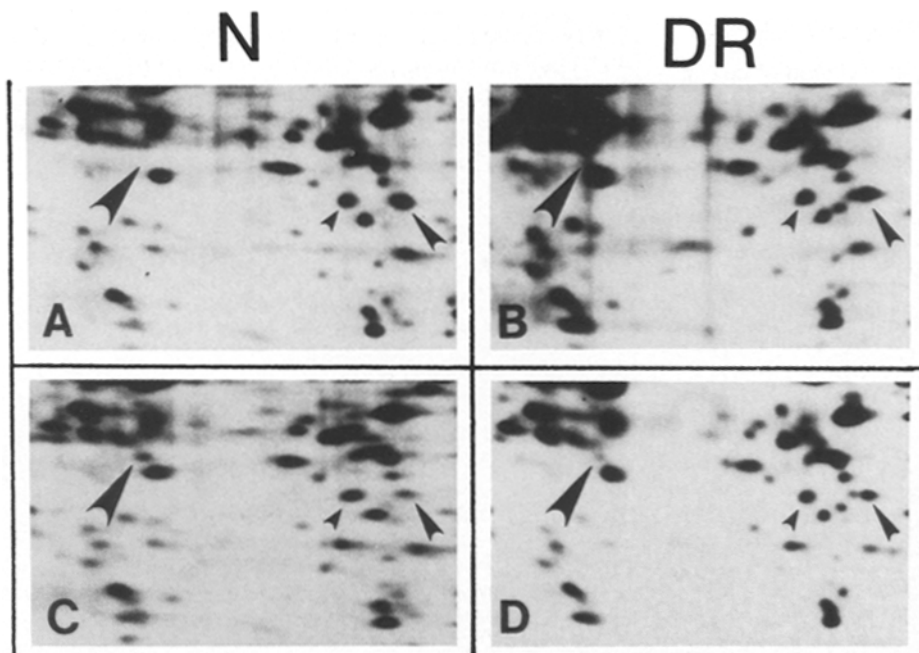


**Figure 3.** Phosphoamino acid analysis of pp42A and pp41 from PDGF-stimulated, naive, and PKC-deficient Swiss 3T3 cells. Naive and PKC-deficient cells were labeled with  $H_3^{32}PO_4$  (2.5 mCi/ml), incubated with PDGF (10 ng/ml) for 10 min at 37°C, lysed, and analyzed by two-dimensional electrophoresis. pp42A and pp41 were excised from replicate gels, the proteins extracted, and acid hydrolyzed. The resulting phosphoamino acids were separated via two-dimensional thin layer electrophoresis and detected by autoradiography as described in Materials and Methods. (A) pp42A, naive cells; (B) pp42A, PKC-deficient cells; (C) pp41, naive cells; (D) pp41, PKC-deficient cells. The positions of phosphoamino acid standards, visualized by ninhydrin staining, are indicated by S, T, and Y (phosphoserine, phosphothreonine, and phosphotyrosine, respectively). The origin was to the lower right of each panel and is not shown.

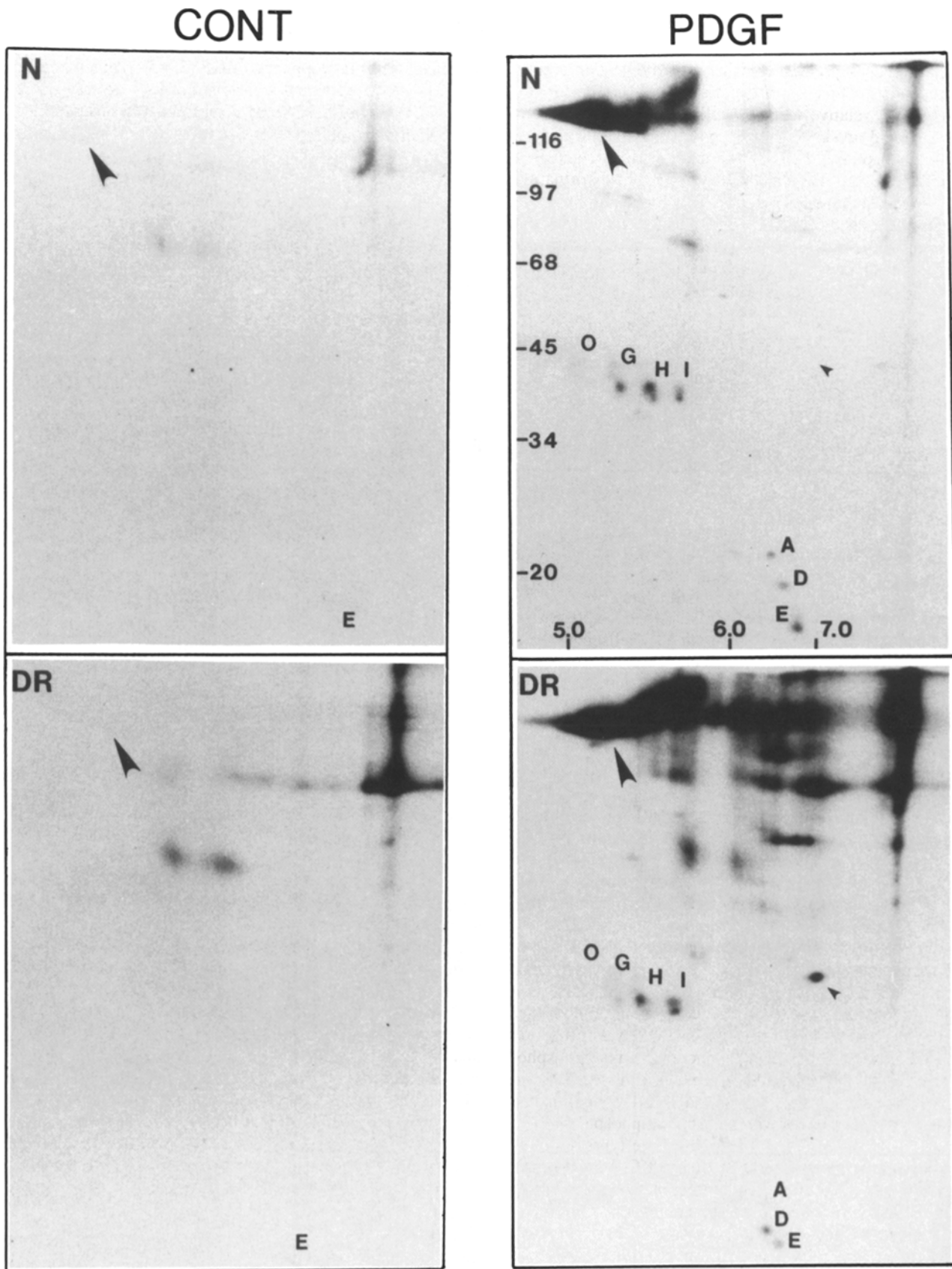
parable quantities of substrate protein (p42), the stoichiometry of phosphorylation of this protein is reduced in PKC-deficient cells.

#### **Other Tyrosine Phosphorylation Events in PKC-deficient Cells**

To what extent are other early growth factor-induced tyrosine phosphorylation events, aside from p42 tyrosine phosphorylation, altered in PKC-deficient cells? We addressed this question by comparing antiphosphotyrosine immunoprecipitates of control and PDGF-stimulated, naive and PKC-deficient Swiss 3T3 cells (Fig. 5). In naive cells, PDGF stimulated the phosphorylation of numerous phosphoproteins including the PDGF receptor (large arrowhead) and seven unidentified phosphoproteins (A, D, E, O, G, H, I) (Fig. 5, top right panel). pp42A and pp45A were not detected (see Discussion). These proteins were much less radioactive in immunoprecipitates from control cells (Fig. 5, top left panel). All immunoprecipitated proteins analyzed contained phosphotyrosine as a major phosphorylated amino acid (not shown). Mixtures of the PDGF-treated total cell lysates (typically containing  $2 \times 10^4$  cells) and antiphosphotyrosine immunoprecipitates (from  $9.2 \times 10^5$  cells) revealed that, with the exception of the PDGF receptor, most other substrates were too minor to be detected on two-dimensional gels of total cell lysates (not shown). The immunoprecipitates of PDGF-treated PKC-deficient cells were overall quite similar to immunoprecipitates of PDGF-treated naive cells (Fig. 5, top and bottom righthand panels). One surprising difference between naive and PKC-deficient samples was the presence of pp42A (small arrowhead), in greater amounts in immunoprecipitates from PDGF-treated, PKC-deficient cells than in PDGF-treated, naive cells (Fig. 5, top and bottom righthand panels). This is the opposite of what was observed in total phosphoproteins of identically treated cells (Fig. 2B).



**Figure 4.** Abundance of p42 in naive and PKC-deficient cells. Naive (N) and PKC-deficient (DR) Swiss 3T3 cells were labeled with 200  $\mu Ci/ml$  [ $^{35}S$ ]methionine for 4 h, incubated with (C and D) or without (A and B) PDGF for 10 min at 37°C, lysed, and analyzed by two-dimensional electrophoresis as in Fig. 1, except IEF was performed with mixed pH 6–8 and pH 8–10 ampholytes, and gels were impregnated with 2,5-diphenyloxazole before fluorography. Large arrowheads point to the position of pp42A (identified by co-migration with  $^{32}P$ -labeled pp42A [data not shown]). Medium-sized arrowheads mark p42, (identified previously [12]). Small arrowheads point to a reference protein.



**Figure 5.** Antiphosphotyrosine immunoprecipitates of PDGF-stimulated naive and PKC-deficient cells. Both naive (*N*) and PKC-deficient (*DR*) Swiss 3T3 cells were incubated without (*CONT*) or with PDGF (10 ng/ml) for 10 min at 37°C, lysed, and immunoprecipitated with a monoclonal antibody to phosphotyrosine. Aliquots were normalized on the basis of cell number and analyzed by two-dimensional electrophoresis as in Fig. 1. The pH of the IEF gels are indicated along the bottom, while molecular mass in kilodaltons is designated along the left side of the top right panel. Large arrowheads point to the PDGF receptor (19), small arrowheads mark the position of pp42A, and the letters mark unidentified PDGF-inducible phosphoproteins.



Note that the immunoprecipitate of unstimulated PKC-deficient cells did not contain pp42 (Fig. 5, bottom left panel).

Additional evidence that tyrosine phosphorylation events, other than p42 phosphorylation, are unaffected in PKC-deficient cells relative to naive cells is provided by phosphoamino acid analysis of pp41. p41 is phosphorylated on tyrosine in response to PDGF stimulation (Fig. 1 and Fig. 3 C; reference 13). The extent of tyrosine phosphorylation of p41 is similar in PDGF-treated PKC-deficient cells and PDGF-treated naive cells (Fig. 3, C and D). Thus, with the exception of p42 and p45, PKC-deficient cells retain their ability to phosphorylate proteins on tyrosine in response to PDGF.

## Discussion

We have shown that numerous agents that stimulate phosphorylation of the PKC substrate, p80, also stimulate p42 phosphorylation. In addition, cells must possess functionally active PKC to mediate growth factor-induced p42 tyrosine phosphorylation. These results suggest that the enzyme responsible for tyrosine phosphorylation of p42 is dependent on PKC. J. Vila and M. Weber have also observed that mitogen-induced p42 phosphorylation is greatly reduced in PKC-deficient chick embryo fibroblasts (manuscript submitted for publication).

In addition to directly measuring PKC activity, we examined the extent of p80 phosphorylation. The disappearance of p80 phosphorylation in PKC-deficient cells correlated with the absence of PKC enzymatic activity in such cells, and is consistent with the observations of numerous other groups (1, 5, 6, 28, 41). It is conceivable that prolonged exposure of cells to TPA results in alterations in addition to a PKC deficiency; however, we have found that with the exception of the inhibited tyrosine phosphorylation of p42 (Fig. 2 B, 3, A and B; Weber, M., personal communication), many tyrosine phosphorylation events proceed in PKC-deficient cells much as they do in naive cells (Fig. 3, C and D, and Fig. 5).

Curiously, pp42A and pp45A are poorly precipitated by antiphosphotyrosine antibodies. Only ~1% of pp42A is precipitated (unpublished data, compare Figs. 1 and 5). It is possible that the phosphotyrosine in pp42 is masked by secondary structure, although the immunoprecipitability of pp42 is not increased by boiling in SDS before immunoprecipitation (unpublished results). Such results raise the possibility that pp42 may be modified via a phosphodiester linkage to tyrosine; however, this is unlikely because the phosphate in pp42 is unaffected by incubation with phosphodiesterase, and is labile to phosphatase treatment (unpublished results). Even though pp42A from naive, PDGF-stimulated cells is poorly immunoprecipitated by antiphosphotyrosine antibodies, the small amount of p42 whose tyrosine phosphorylation is stimulated by PDGF in PKC-deficient cells (Fig. 2) is very efficiently immunoprecipitated (Fig. 5; three independent experiments). A likely explanation of this result is that the site of tyrosine phosphorylation of p42 in naive and PKC-deficient cells is not the same, and that only p42 with phosphate at the PKC-deficient site is efficiently recognized by the antiphosphotyrosine antibody.

Unlike mitogens such as PDGF, TPA, or calf serum, the ability of EGF to stimulate PKC appears somewhat variable. In some cell types EGF clearly activates PKC, whereas in others

it does so weakly or not at all (6, 28, 30). In addition, increases in phosphoinositide turnover are only detected in some cell types in response to EGF (3, 43). Thus it appears that EGF-induced signal transduction does not always require PKC activation. We have reproducibly observed that EGF stimulates p80 phosphorylation in Swiss 3T3 cells. That PKC mediates EGF-induced tyrosine phosphorylation of p42 in these cells is further suggested by the observation that, in PKC-deficient cells, EGF-induced phosphorylation of both p80 and p42 is dramatically reduced (unpublished results). Perhaps in some cell types the EGF stimulation of phosphoinositide turnover is a local phenomenon, and DAG accumulation is insufficient to activate a large fraction of PKC molecules, but could be sufficient to trigger other events including p42 tyrosine phosphorylation.

PDGF can initiate proliferation in PKC-deficient human fibroblasts (17) and Swiss 3T3 cells (our unpublished results). This suggests either that PKC does not participate in growth signal relay or that there are alternate or "backup" pathways. However, there is a low level of residual PKC in PKC-deficient cells (45). Indeed, PKC-deficient cells have increased basal p80 phosphorylation, and incubation with PDGF results in a slight stimulation of p80 phosphorylation and p42 phosphorylation at serine (Figs. 2 and 3; Table II). Thus it is conceivable that the low level of PDGF-induced p42 serine phosphorylation in PKC-deficient cells is sufficient to relay the mitogenic signal. Alternatively, the tyrosine phosphorylation of p42 that occurs transiently at the start of the down regulation procedure may place the cells in a different state, from which PDGF can stimulate mitogenesis without PKC involvement. Whether the phosphorylation of p42 on tyrosine constitutes a significant event of a relay network, which can be circumvented when PKC is nonfunctional, remains to be determined.

These studies provide a possible explanation to the following discrepancy. Infection of avian cells with transforming retroviruses encoding protein tyrosine kinases (*v-fps*, *v-src*, *v-erb-B*) results in p42 tyrosine phosphorylation (10, 11, 22), but in mammalian cell lines transformed with *v-fps*, *v-src*, *v-fgr*, *v-fes*, or *v-abl*, p42 is not phosphorylated (11, 14, and unpublished results of J. A. Cooper and T. Hunter). The absence of phosphorylated p42 in retrovirally transformed mammalian cells may be due to the failure of these retroviruses to chronically augment PKC activity in mammalian cells.

Perhaps the most interesting implication of these studies is that tyrosine phosphorylation of p42, initiated by mitogens such as PDGF, occurs via a cascade of events. This cascade includes at least three kinases: the PDGF protein-tyrosine kinase, PKC, and the PKC-dependent p42-specific kinase. In vitro attempts to identify and characterize the last member of this cascade (i.e., the PKC-dependent, p42-specific enzyme) are in progress.

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