Platelet-derived growth factor (PDGF) stimulated the phosphorylation of a 170,000-Mr protein in membranes prepared from parental and several variant lines of Swiss 3T3 cells as well as from diploid human fibroblasts. The intensity of the phosphorylation of the 170,000-Mr protein paralleled the number of PDGF receptors present on the various cells. The enhanced phosphorylation was the result of an increase in the amount of phosphoserine and phosphotyrosine present in the 170,000-Mr protein. PDGF-stimulated phosphorylation of the 170,000-Mr protein was rapid and showed a dose response to PDGF. Down regulation of the PDGF receptor led to a rapid loss in the PDGF-stimulated phosphorylation of the 170,000-Mr protein. In Swiss 3T3 cell membranes, PDGF as well as epidermal growth factor stimulated the phosphorylation of a synthetic, tyrosine-containing peptide. The effects of the two growth factors were additive, indicating that PDGF and epidermal growth factor stimulate peptide phosphorylation through distinct receptors.

PDGF is one of the major mitogens found in serum (1-3). This growth factor, which is stored in platelets and released during degranulation, stimulates DNA synthesis and cell division in connective tissue cells (1, 2, 4). PDGF has been highly purified in a number of laboratories (5-8). It exhibits a molecular weight of approximately 30,000 and is comprised of two peptide chains with molecular weights of 17,000 and 14,000 linked by disulfide bonds (6-8).

Radioiodination studies using [125I]-PDGF indicate that this growth factor binds with high affinity to a discrete number of receptor sites on 3T3 cells, human foreskin fibroblasts, and several other cell types (9, 10). The molecular weight of the PDGF receptor appears to be around 165,000 based on data obtained from experiments in which [125I]-PDGF was covalently cross-linked to cell surface proteins (11).

Recent studies on the mechanism of action of another mitogen, EGF, have demonstrated that, in A431 cells, EGF stimulates the phosphorylation on a tyrosine residue of a 150,000 to 170,000-Mr protein which appears to be the EGF receptor (12-14). Exogenous proteins added to the A431 membranes, PDGF as well as epidermal growth factor stimulated the phosphorylation of a synthetic, tyrosine-containing peptide. The effects of the two growth factors were additive, indicating that PDGF and epidermal growth factor stimulate peptide phosphorylation through distinct receptors.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Materials and Methods

Cell Culture—The Swiss 3T3 cell variant NR-6 which does not express EGF receptors (25) was obtained from H. Herschman (University of California, Los Angeles, CA). The Swiss 3T3 cell variants PP2 and CI-1 expressing low levels of PDGF receptors were selected as previously described (10). Variant PP2 had partially reverted toward expression of the parental number of PDGF receptors at the time these experiments were performed. Swiss 3T3 and their SV40 transformants were obtained from A. Vogel (University of Washington, Seattle, WA). The adult human diploid fibroblasts were obtained essentially as described by Baker et al. (26), and were used in the 6th through 9th passages. Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% calf serum (GIBCO) for the Swiss 3T3 and NR-6 variants, or with 10% calf serum for the human diploid fibroblasts cells, or with 5% calf serum plus 5 ng/ml of porcine insulin (Sigma) and 5 ng/ml of EGF (Collaborative Research) for the PP2 and CI-1 variants. For the experiments reported here, all cultures were grown to confluence in 150-mm Integridic culture dishes (Falcon) in the media described above, then incubated for 2-4 days in Dulbecco's modified Eagle's medium supplemented with 2% calf serum from which the PDGF had been removed as described by Vogel et al. (27).

Membranes—Membranes were prepared by scraping the cells from 150-mm dishes in the presence of 20 ml of 5 mM HEPES, pH 7.4, 2 mM MgCl2, and 5 mM d-mercaptoethanol. The cells were then homogenized with 20 strokes in a Dounce homogenizer using a tight-fitting pestle. The homogenate was centrifuged at 18,000 rpm for 30 min, and the pellet reusupended for assay by homogenization in 40 mM HEPES pH 7.4, 206 mM NaCl, 0.4 mM Triton X-100.

Reagents—Highly purified human PDGF was prepared as previously described (8). EGF was a gift of Stanley Cohen (Vanderbilt University, Nashville, TN). The peptide with the sequence Arg-Arg-Glu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Ala-Ary-Gly, synthesized and purified as described elsewhere (28), was the gift of John Casnellie (University of Washington, Seattle, WA). Phosphoserine and phosphotyrosine were purchased from Sigma. Phosphotyrosine was prepared according to the method of Rothberg et al. (29). [1-13C]PDATP and [125I]EGF were from New England Nuclear.
PDGF-stimulated Phosphorylation

Assays—Membrane phosphorylation assays were carried out in a total volume of 30 μl (in final concentrations): 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM Tris-HCl, 0.1% Triton X-100, 10 mM MnCl₂, 40 μM adenosine 5′-(β,γ-imino)triphosphate, 5 mM p-nitrophenyl phosphate, 10 μM [γ-32P]ATP (10,000–20,000 cpm/pmol) and approximately 30–40 μg of membrane protein. Based on whole cell binding studies, this amount of protein contains 90–135 fmol of PDGF binding activity for NR6-S3T3 cell membranes. Adenosine 5′-(β,γ-imino)triphosphate was included to reduce nonspecific hydrolysis of ATP; p-nitrophenyl phosphate was added to inhibit protein phosphatases. Unless otherwise indicated, assays contained 70 nm PDGF or 540 nm EGF + 5 μg of carrier bovine serum albumin or 5 μg of bovine serum albumin for controls. Assays were started by adducing the membrane suspension and incubated at 30 °C for the time stated in the figure legends. The reactions were terminated by addition of 30 μl of SDS buffer (2.5% SDS, 100 mM dithiothreitol, 125 mM Tris, pH 6.7, 2.5 mM EDTA, 50% glycerol) and subsequently boiled for 3 min prior to application to a 5 to 10% polyacrylamide gel (30). Gels were stained to locate standard proteins, dried, and autoradiographed for 8 to 24 h. The molecular weight standards used were: myosin (200,000); β-galactosidase (116,000); phosphorylase b (92,000); bovine serum albumin (68,000); ovalbumin (44,000); and chymotrypsinogen (25,000).

Two-dimensional polyacrylamide gel electrophoresis was performed according to O'Farrell (31).

Peptide phosphorylation assays were performed under the same conditions as the membrane phosphorylation assays with the exception that synthetic peptides were added to the reaction mixture at a concentration of 10 μM. The reactions were terminated by addition of 50 μl of 0.1% trichloroacetic acid. The tubes were centrifuged to pellet the precipitated proteins, and a 40-μl aliquot of the supernatant was spotted on phosphocellulose paper, washed four times with 0.1 M acetic acid and hydrolyzed for 2 h at 110 °C. The 32P-labeled phosphoamino acids were separated by high voltage electrophoresis at pH 3.6 for 3000 V, and were identified by co-migration with phosphoamino acid standards which were detected with ninhydrin.

RESULTS

Characteristics of PDGF-stimulated Membrane Protein Phosphorylation—Membranes prepared from a variety of different cell types were incubated with [γ-32P]ATP in the absence or presence of PDGF and analyzed as described under “Materials and Methods.” The results are shown in Fig. 1. Under these conditions, a number of proteins were phosphorylated in all of the membranes tested. However, the phosphorylation of a single protein with a molecular weight of approximately 170,000 was specifically increased in the presence of PDGF. Occasionally, PDGF stimulated the phosphorylation of a 130,000-M₉ protein (not shown). Because this latter result was not consistently observed, this finding was not further examined. The increase in phosphorylation of the 170,000-M₉ protein was observed in 3T3 cell lines as well as in diploid human fibroblasts. As can be seen from Fig. 1, the intensity of the phosphorylated 170,000-M₉ band varies directly with the number of PDGF receptors present on the cells as judged by 125I-PDGF binding to parallel cell cultures. Thus, the PF2 line of 3T3 cells, which exhibits a reduced number of PDGF receptors, showed a weak response to PDGF and the CI-1 line of 3T3 cells, which possesses one sixth as many PDGF receptors as the PF2 line, exhibited no detectable response to PDGF when examined for the stimulation of

170,000-M₉ protein phosphorylation. In addition, a line of 3T3 cells found to have a reduced number of PDGF receptors following transformation by SV40 also showed a diminished response to PDGF in the phosphorylation assay. The response to PDGF does not appear to correlate with the presence or absence of EGF receptors since PF2 and CI-1 3T3 cells both possess EGF receptors (data not shown) but did not respond to PDGF. Furthermore, EGF receptor-negative NR-6 3T3 cells showed an increase in the phosphorylation of the 170,000-M₉ protein in response to PDGF. Because NR-6 3T3 cells contain PDGF receptors but no EGF receptors, this line was selected for further study.

As shown in Fig. 2A, the PDGF-stimulated phosphorylation of the 170,000-M₉ protein in NR6-3T3 cell membranes was rapid. Phosphorylation of the band was apparent after 1 min in the presence of PDGF and was still increasing at 30 min. The time course was biphasic, showing an early, rapid rate of phosphorylation followed by a slower rate of 32P incorporation into the 170,000-M₉ protein. Dephosphorylation of the 170,000-M₉ band, observed after addition of unlabeled ATP and EDTA, was slow exhibiting a t½ of about 60 min (Fig. 2B).

Phosphoamino acid analyses of the phosphorylated 170,000-M₉ protein for 3 min (the early phase of the time course) for 15 min (the later phase of the time course) were performed. The results, shown in Fig. 3, indicated that at both time points, PDGF stimulated the incorporation of 32P into phosphoserine and phosphotyrosine. In other experiments, traces of phosphothreonine were found in the 170,000-M₉ protein and the content of this phosphoamino acid was also increased in the presence of PDGF.

The fact that phosphoserine, phosphotyrosine, and phosphothreonine are present in the 170,000-M₉ protein indicates

2 D. F. Bowen-Pope, A. Vogel, and R. Ross, unpublished observations.
PDGF-stimulated Phosphorylation

A

PDGF + + + + + + +
Time (min) 1 2 5 15 30

B

PDGF + + + + + + +
Time (min) 0 2 5 15 30 60

Fig. 2. Time course of the PDGF-stimulated phosphorylation and the dephosphorylation of the 170,000-Mr protein in NR-6 3T3 cell membranes. A, NR-6 3T3 cell membranes were incubated with [γ-32P]ATP for the times indicated in the absence or presence of PDGF. The plotted data points represent the difference in the amount of phosphorylation observed in the presence of PDGF and that observed in the absence of PDGF as calculated from densitometric scans of the autoradiogram. B, NR-6 3T3 cell membranes were incubated with [γ-32P]ATP in the absence or presence of PDGF. The phosphatase inhibitor, p-nitrophenyl phosphate was not included in these reaction mixtures. After 15 min (at t = 0), unlabeled ATP and EDTA were added to the reaction mixtures at final concentrations of 1 and 10 mM, respectively, and the incubations were continued for the times indicated. The plotted data points are as in A. In A and B, only the portion of the gel containing the 170,000-Mr protein is shown.

Fig. 3. Phosphoamino acid analysis of the 170,000-Mr protein phosphorylated in the absence or presence of PDGF. NR-6 3T3 cell membranes were incubated with [γ-32P]ATP for 3 min or 15 min in the absence or presence of PDGF. Following separation of the phosphorylated proteins by polyacrylamide gel electrophoresis, the 170,000-Mr protein was excised and analyzed for phosphoamino acids as described under "Materials and Methods." That this species is phosphorylated on multiple sites. This conclusion is supported by the results shown in Fig. 4. 3T3 membranes were phosphorylated in the absence or presence of PDGF as usual and subjected to two-dimensional polyacrylamide gel electrophoresis. Isoelectric focussing was performed in the first dimension followed by SDS-gel electrophoresis in the second dimension. As expected, PDGF stimulated the phosphorylation of a protein(s) with a molecular weight of approximately 170,000. Both in the absence and presence of PDGF, the phosphorylated material in the 170,000-Mr region was comprised of several distinct species that differed in charge, possibly due to differences in the degree of phosphorylation of a single protein. The pl of the protein(s) that showed an increase in phosphorylation in response to PDGF was 5.3 to 5.5.

To obtain an estimate of the amount of phosphorylation of the 170,000-Mr protein relative to the number of PDGF

Fig. 4. Two-dimensional polyacrylamide gel analysis of the phosphorylation of NR-6 3T3 cell membrane proteins in the absence and presence of PDGF. NR-6 3T3 cell membranes were phosphorylated with [γ-32P]ATP in the absence (top) or presence (bottom) of PDGF for 3 min as described under "Materials and Methods." Assays were stopped by the addition of SDS-containing buffer and boiled for 3 min. The sample was prepared for isoelectric focussing and SDS-gel electrophoresis according to O'Farrell (31). The pH gradient in the first (horizontal) dimension was from pH 5 to pH 8. A 6% polyacrylamide gel was run in the second (vertical) dimension.
receptors present in the assays, NR6-3T3 cell membranes were phosphorylated in the absence or presence of PDGF and the proteins separated by SDS-gel electrophoresis. The 170,000-Mr bands were excised from the gel and counted for 32P. At an ATP concentration of 50 μM, PDGF stimulated the incorporation of 1065 cpm of 32P into the 170,000-Mr band. Based on estimates of the number of PDGF receptors present (see "Materials and Methods") and the specific activity of the ATP (6,000 cpm/pmol), this corresponds to 1–2 mol of phosphate incorporated/mol of PDGF binding activity.

The cAMP-dependent protein kinase does not appear to be involved in the serine phosphorylation of the 170,000-M, protein since addition of purified catalytic subunit or inclusion of the specific heat-stable inhibitor of this enzyme, did not alter the phosphorylation of the 170,000-M, protein (data not shown).

Fig. 5A shows the dose response to PDGF for the stimulation of the phosphorylation of the 170,000-M, protein in NR6-3T3 cell membranes. Densitometer scans of the autoradiogram indicated that at the highest dose, PDGF stimulated a 3- to 4-fold increase in the phosphorylation of the 170,000-M, band. Half-maximal stimulation of phosphorylation occurred at approximately 3 nM (Fig. 5B).

PDGF-stimulated phosphorylation of the 170,000-M, protein in NR6-3T3 cell membranes exhibited a requirement for divalent cations. The dose response to Mn2+ is shown in Fig. 6. Increasing the concentration of Mn2+ from 1 mM to 20 mM increased the PDGF-stimulated phosphorylation of the 170,000-M, band. At the highest concentration of Mn2+, 20 mM, a significant increase in the basal phosphorylation of the 170,000-M, band was observed. Thus, the amount of PDGF-specific phosphorylation was reduced relative to that observed with 10 mM Mn2+. All assays were therefore performed using 10 mM Mn2+. At high concentrations, Mg2+ also supported PDGF-stimulated phosphorylation of the 170,000-M, band. However, the amount of 32P incorporated into this protein in the presence of Mg2+ was significantly less than that observed using Mn2+ (not shown).

Down Regulation of the Response to PDGF—Incubation of whole cells with high concentrations of PDGF leads to a decrease in the capacity of the cells to bind PDGF (9, 10, 32), a phenomenon known as down-regulation. To examine the effect of down-regulation on PDGF-stimulated 170,000-M, protein phosphorylation, NR6-3T3 cells were preincubated with 25 ng/ml of PDGF for 0 to 7 h as described in the legend to Fig. 7. Cultures were subsequently washed and membranes were prepared and assayed for phosphorylation of the 170,000-M, protein in the absence and presence of PDGF. Total cell-associated PDGF was measured in parallel cultures preincubated with 25 ng/ml of 125I-PDGF. Unoccupied cell-surface receptors were assayed by 125I-PDGF postlabeling of cultures preincubated for various times with 25 ng/ml of unlabeled PDGF.

As shown in Fig. 7A, a 1-h preincubation of the NR6-3T3 cells with PDGF resulted in a significant reduction in the level of phosphorylation of the 170,000-M, protein stimulated by PDGF in the membranes. Densitometer scans of the autoradiogram (not shown) indicated that the decrement in PDGF-stimulated phosphorylation of the 170,000-M, protein was approximately 90%. After a 3-h preincubation of the cells with PDGF, no increase in the 170,000-M, protein phosphorylation in response to PDGF could be detected in the membranes. The alteration in phosphorylation in the membranes following preincubation of the cells with PDGF appeared to

---

**Fig. 5. Dose response of PDGF for the stimulation of 170,000-M, protein phosphorylation in NR-6 3T3 cell membranes.** A, NR-6-3T3 cell membranes were incubated with [γ-32P]ATP for 3 min in the absence (lane a) or presence (lanes b–g) of PDGF and the proteins separated by polyacrylamide gel electrophoresis. The molar concentrations of PDGF used were: lane b, 8.6 × 10−10; lane c, 2.5 × 10−9; lane d, 4.0 × 10−8; lane e, 8 × 10−8; lane f, 2.3 × 10−7; lane g, 7 × 10−7; and, lane h, 1.4 × 10−6. B, the autoradiogram from A was scanned with a densitometer and the data plotted as described in the legend to Fig. 2. Molecular weight standards are shown × 103.

**Fig. 6. Dose response to Mn2+ of the PDGF-stimulated phosphorylation of the 170,000-M, protein in NR-6 3T3 cell membranes.** NR-6-3T3 cell membranes were incubated with [γ-32P]ATP for 3 min in the absence or presence of PDGF and the indicated concentration of MnCl2. Molecular weight standards are shown × 103.
be specific for the 170,000-M₆ protein since no increase or decrease in the phosphorylation of other proteins was observed. Fig. 7B demonstrates that preincubation of NR6-3T3 cells with PDGF led to a decrease in unoccupied cell surface PDGF receptors. Both the extent and the time course of the decrease in unoccupied cell-surface receptors were similar to that observed for the fall in 170,000-M₆ protein phosphorylation. Incubation of the cells with 125I-PDGF for increasing lengths of time resulted in a decline in total cell-associated radioactivity (Fig. 7B). However, the time course of the decrease in total cell-associated 125I radioactivity was much slower than that observed for the declines in PDGF-stimulated phosphorylation and unoccupied cell surface PDGF receptors.

Comparison of PDGF- and EGF-stimulated Phosphorylation of Membrane Proteins and a Synthetic Peptide—Swiss 3T3 cells contain both PDGF receptors (120,000 receptors/cell) and EGF receptors (60,000–100,000 receptors/cell). Therefore, membranes prepared from these cells were used in studies comparing the effects of these two growth factors on phosphorylation. As shown in Fig. 8, in Swiss 3T3 cell membranes, PDGF increased the phosphorylation of a protein with a M₆ ~170,000. By contrast, EGF stimulated the phosphorylation of a protein with M₆ 160,000. The response of the membranes to PDGF was greater than that observed with EGF, consistent with the larger number of PDGF receptors in these membranes. These reactions were carried out at 0 °C since the EGF response could not be detected at 30 °C.

We have previously demonstrated that in A431 (15) and 3T3 (33) cell membranes, EGF stimulates the phosphorylation of a synthetic, tyrosine-containing peptide. The sequence of the peptide is Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly and is similar to the sequence surrounding the site of tyrosine phosphorylation in pp60⁺⁺⁺, the transforming protein of Rous sarcoma virus (34–36). Because PDGF stimulates the phosphorylation of an endogenous membrane pro-
phosphorylation of a 130,000-Mr protein. Since this result was not consistently obtained, it is possible that the 130,000-Mr protein may be generated through proteolysis of the 170,000-Mr species. We find that PDGF increases the incorporation of phosphate into serine, tyrosine, and occasionally threonine residues in the 170,000-Mr protein. The heterogeneity of the phosphoamino acids present in the 170,000-Mr band suggests that this protein serves as a substrate for more than one protein kinase. Phosphorylation on both serine and tyrosine residues also occurs in pp60v-src. In that case, the tyrosine phosphate appears to be the product of an autophosphorylation reaction while the serine phosphorylation is catalyzed by a cAMP-dependent protein kinase (18, 37-39). When purified catalytic subunit of cAMP-dependent protein kinase was added to 3T3 cell membranes, no apparent increase in the phosphorylation of the 170,000-Mr band was observed. No decrease in phosphorylation was observed when the low molecular weight inhibitor of the cAMP-dependent protein kinase was included in the assays. Thus, it is unlikely that the cAMP-dependent protein kinase plays a role in the phosphorylation of the 170,000-Mr protein.

In all of the cell lines used in these studies, the molecular weight of the protein phosphorylated in the presence of PDGF was consistently 170,000. The fact that the apparent size of the phosphorylated protein was conserved from murine to human cell types suggests that the 170,000-Mr protein may play an important role in the cell, possibly as the cell-surface receptor for PDGF. EGFP stimulates the phosphorylation of its receptor on a tyrosine residue (12-14). By analogy, PDGF might also be expected to enhance the phosphorylation of the PDGF receptor on a tyrosine.

Several lines of evidence are consistent with the hypothesis that the 170,000-Mr protein phosphorylated in response to PDGF is the PDGF receptor. First, 125I-PDGF binds to, and can be specifically cross-linked to, a protein of about 164,000 Da present on the surface of 3T3 cells (19). In addition, in these studies, the intensity of the phosphorylation of the 170,000-Mr protein in a series of different cell types roughly paralleled the number of PDGF receptors measured by 125I-PDGF binding to those cells. Finally, the phosphorylation of the 170,000-Mr protein decreased in parallel with the binding of PDGF to its receptor in down-regulation experiments. The Characteristics of the Phosphorylation Response—The phosphorylation of the 170,000-Mr protein showed a dose response to PDGF in NR-6 3T3 cells with half-maximal activation at approximately 8 nM. This is somewhat higher than the observed Kd for 125I-PDGF binding in these cells (10). However, the binding experiments were performed on intact cells at 0 °C, while the phosphorylation experiments were carried out at 30 °C with membrane preparations, so the values for the binding Kd and half-maximal activation for phosphorylation are not directly comparable.

An increase in the phosphorylation of the 170,000-Mr protein occurred within 1 min after addition of PDGF. A small increase could occasionally be seen after 30 s. Thus, this phosphorylation appears to be a very early event triggered by PDGF. The PDGF-stimulated phosphorylation of the 170,000-Mr band was linear for approximately 5 min. The different rate of phosphorylation observed after 5 min may be due to the slower phosphorylation of a second site or merely to depletion of one or more of the substrates. Dephosphorylation of the 170,000-Mr protein was slow, with a t1/2 of about 60 min. These data are in marked contrast to the findings with the EGF-stimulated phosphorylation of A431 membranes, where dephosphorylation occurs with a t1/2 of 5 min (13). This observation may reflect differences in phosphatase activities present in NR-6 and A431 cell membranes. The NR-6 cell membranes showed greater PDGF-stimu-

![Image](http://www.jbc.org/)

**Table 1**

| Condition          | 32P incorporated | pmol/min/mg protein |
|--------------------|-------------------|---------------------|
| No growth factor   | 1.4               |                     |
| +PDGF              | 3.5               |                     |
| +EGF               | 4.7               |                     |
| +PDGF + EGF        | 6.6               |                     |

**DISCUSSION**

*The Nature of the 170,000-Mr Protein—* The data presented here confirm and extend the findings of Ek et al. (22, 23) and Nishimura et al. (24) concerning the stimulation of phosphorylation by PDGF of a high molecular weight protein in cell membranes. In both 3T3 and human fibroblast membranes, PDGF enhanced the phosphorylation of a protein with an apparent Mr of 170,000. Similar to the findings of Ek et al. (22), we occasionally observed that PDGF stimulated the phosphorylation of a 130,000-Mr protein. Since this result
lated phosphorylation of the 170,000-Mr protein in the presence of Mn++ than in the presence of Mg++. The preference for Mn++ in these membranes may be due, in part, to the ability of this divalent cation to inhibit phosphatases present in the membranes (28).

Down-regulation of PDGF-stimulated Phosphorylation and PDGF Binding Occur in Parallel—PDGF has previously been shown to induce down-regulation of its receptor in 3T3 cells (9, 10, 32). In the present experiments, preincubation of 3T3 cells for up to 7 h with a high dose of PDGF or [3H]-PDGF led to decreases in: (i) total cell-associated [3H]-PDGF; (ii) unoccupied cell-surface PDGF receptors; and, (iii) the amount of phosphorylation of the 170,000-Mr protein. The decrease in phosphorylation was specific for the 170,000-Mr protein and paralleled the decline in the number of unoccupied PDGF receptors. The decrease in these two parameters was rapid; a 90% reduction occurred after a 1-h preincubation with PDGF. By contrast, the decline in total cell-associated radioactivity was relatively slow, exhibiting a t1/2 of 3 to 4 h. The fact that the phosphorylation of only the 170,000-Mr protein was altered by down-regulation of the PDGF receptor supports the idea that this phosphorylation is a specific response to PDGF. The correlation of the decrease in phosphorylation with the decrease in free, cell-surface PDGF receptors suggests that phosphorylation of the 170,000-Mr protein in membranes is only stimulated through unoccupied growth factor receptors. This may be a consequence of the rapidity of the phosphorylation of the 170,000-Mr protein. Occupation of the PDGF receptor by PDGF during the preincubation may lead to the immediate phosphorylation of the 170,000-Mr protein in the cells. Since this phosphorylation would occur with unlabeled endogenous ATP, these sites would be blocked and would not be measured during subsequent labeling of the membranes with [3H]ATP. Alternatively if the 170,000-Mr protein is the PDGF receptor, occupied PDGF receptors may be internalized rapidly in a form that cannot be phosphorylated or recovered with the membrane fraction as prepared for these studies. Such receptor internalization apparently accounts for the down-regulation induced by EGF (40, 41) and insulin (42, 43). The rapid loss of surface PDGF receptors in conjunction with the slow decline in cell-associated radioactivity observed in the present experiments is consistent with receptor internalization as a mechanism for PDGF-induced down-regulation.

Similarities and Differences between PDGF- and EGF-stimulated Phosphorylation—In 3T3 cell membranes which respond to both growth factors, PDGF and EGF stimulate the phosphorylation of distinct proteins. PDGF stimulates the phosphorylation of a 170,000-Mr protein whereas EGF stimulates the phosphorylation of a 160,000-Mr protein. Similar findings have been reported by Nishimura et al. (24). It is interesting to note that this response to EGF could be detected when the assays were performed at 0°C but not at 30°C. The PDGF-stimulated response did not show a similar temperature dependence. Since phosphatases and proteases are inhibited at lower temperatures, this observation suggests that the EGF-stimulated phosphorylation of the 160,000-Mr protein in these membranes may be more sensitive to the actions of such enzymes than is the PDGF-stimulated phosphorylation of the 170,000-Mr protein. The fact that EGF-stimulated peptide phosphorylation occurs at 30°C indicates that this growth factor-regulated kinase is not inactivated at this temperature.

PDGF was found to stimulate the phosphorylation of a synthetic tyrosine-containing peptide by 3T3 cell membranes. Half-maximal stimulation of peptide phosphorylation by PDGF occurred at 6 nM, a value in good agreement with the value of 3 nM observed for half-maximal stimulation of the 170,000-Mr protein phosphorylation by this growth factor. Previously, EGF had been shown to stimulate the phosphorylation of the same peptide in 3T3 (36) and A431 (15) cell membranes. The effects of PDGF and EGF on peptide phosphorylation in the 3T3 membranes were found to be additive, suggesting that these two growth factors work through independent cell-surface receptors. The finding that PDGF and EGF stimulate the phosphorylation of distinct membrane proteins in 3T3 cell membranes, which may represent the cell-surface receptor for these two growth factors, is consistent with this conclusion. The additivity noted between PDGF- and EGF-stimulated peptide phosphorylation is in sharp contrast to the lack of additivity noted for transforming growth factor and EGF-stimulated peptide phosphorylation in these same membranes (36). Transforming growth factor and EGF are thought to interact with the same EGF receptor (44) and would therefore not be expected to produce an additive response.

Although PDGF and EGF both stimulated peptide phosphorylation in 3T3 cell membranes, the response to EGF was stronger. Since the PDGF- and EGF-stimulated phosphorylation reactions exhibited similar Kms, values for the peptide, the difference in activity appears to be due to differences in the Vmax of the two growth factor-stimulated reactions. This is surprising since these cells possess far more PDGF receptors than EGF receptors/cell and may be indicative of some difference in the specificities of the PDGF- and EGF-stimulated kinases.

Increases in tyrosine phosphorylation have now been associated with three different growth factors, EGF (14), PDGF (22, 24, 45) and insulin (46-48). The exact role of tyrosine phosphorylation in the mechanism of action of growth factors has yet to be elucidated. However, the fact that an increase in the phosphorylation of proteins on serine as well as tyrosine residues was observed in these experiments, as well as by others (45, 49), suggests the possibility that a growth factor-stimulated tyrosine protein kinase might catalyze the phosphorylation and activation of serine (threonine) protein kinases. The diverse phosphorylations carried out by these latter enzymes might then regulate the host of biochemical events triggered by the growth factor. Alternatively, growth factor-induced changes in protein phosphorylation could be accomplished through alterations in the activities of phosphoprotein phosphatases. The ability of PDGF and EGF to stimulate phosphorylation of the same synthetic, tyrosine-containing peptide suggests that these two growth factor-regulated kinases have at least partially overlapping substrate specificity. Activation of a similar, but not identical set of kinases by these two growth factors would explain the similarities and differences in the response of cells to PDGF and EGF.

Acknowledgments—We wish to thank Stephanie Allen for technical assistance.

REFERENCES

1. Ross, R., Glomset, J., Kariya, B., and Harker, L. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1207-1210
2. Kohler, N., and Lipton, A. (1974) Exp. Cell Res. 87, 297-301
3. Westermark, B., and Wasteson, D., and Kennedy, B. B. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7314-7317
9. Hedlin, C. H., Westernmark, B., and Wasteson, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3664–3668
10. Bowen-Pope, D. F., and Ross, R. (1982) J. Biol. Chem. 257, 5161–5171
11. Glenn, K., Bowen-Pope, D. F., and Ross, R. (1982) J. Biol. Chem. 257, 5172–5176
12. Carpenter, G., King, L., Jr., and Cohen, S. (1979) J. Biol. Chem. 254, 4884–4891
13. Cohen, S., Carpenter, G., and King, L., Jr. (1980) J. Biol. Chem. 255, 4834–4842
14. Ushiro, H., and Cohen, S. (1980) J. Biol. Chem. 255, 8363–8365
15. Pike, L. J., Gallis, B., Casnellie, J. E., Bornstein, P., and Krebs, E. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1443–1447
16. Collett, M. S., Purchio, A. F., and Erikson, R. L. (1980) Nature (Lond.) 285, 167–169
17. Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S., and Erikson, R. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3159–3163
18. Hunter, T., and Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1311–1315
19. Levinson, A. D., Oppermann, H., Varmus, H. E., and Bishop, J. M. (1980) J. Biol. Chem. 255, 11973–11980
20. Oppermann, H., Levinson, A. D., Varmus, H. E., Levintow, L., and Bishop, J. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1804–1808
21. Witte, O. N., Dasgupta, A., and Baltimore, D. (1980) Nature (Lond.) 283, 826–831
22. Ek, B., Westernmark, B., Wasteson, A., and Heldin, C.-H. (1982) J. Biol. Chem. 257, 419–420
23. Ek, B., and Hedlin, C.-H. (1982) J. Biol. Chem. 257, 10486–10492
24. Nishimura, J., Huang, J. S., and Deuel, T. F. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4303–4307
25. Pruss, R. M., and Herschman, H. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3918–3921
26. Baker, J. B., Barsh, G. S., Carney, D. H., and Cunningham, D. D. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 1882–1886
27. Vogel, A., Raines, E., Kariya, B., Rivest, M. J., and Ross, R. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2810–2814
28. Casnellie, J. E., Harrison, M. L., Pike, L. J., Hellstrom, K. E., and Krebs, E. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 282–286
29. Rothberg, P. G., Harris, T. J. R., Nomoto, A., and Wimmer, E. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4868–4872
30. Laemmli, U. K. (1970) Nature (Lond.) 227, 680–685
31. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
32. Heldin, C.-H., Wasteson, A., and Westernmark, B. (1982) J. Biol. Chem. 257, 4216–4221
33. Pike, L. J., Marquardt, H., Todaro, G. J., Gallis, B., Casnellie, J. E., Bornstein, P., and Krebs, E. G. (1982) J. Biol. Chem. 257, 14628–14631
34. Czerwiński, A. P., Levinson, A. D., Varmus, H. E., and Bishop, J. M. (1981) Nature (Lond.) 287, 198–203
35. Neil, J. C., Gysdæl, J., Vogt, P. R., and Smart, J. E. (1981) Nature (Lond.) 291, 675–677
36. Smart, J. E., Oppermann, H., Czerwiński, A. P., Purchio, A. F., Erikson, R. L., and Bishop, J. M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6013–6017
37. Collett, M. S., Erikson, E., and Erikson, R. L. (1979) J. Virol. 29, 770–781
38. Shealy, D. J., and Erikson, R. L. (1981) Nature (Lond.) 293, 666–669
39. Purchio, A. F. (1982) J. Virol. 41, 1–7
40. Carpenter, G., and Cohen, S. (1976) J. Cell Biol. 71, 159–171
41. Abasamov, A., Pruss, R. M., and Herschman, H. R. (1978) J. Biol. Chem. 253, 3970–3977
42. Gavin, J. R., Roth, J., Neville, D. M., DeMeyts, P., and Buell, D. N. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 84–88
43. Mott, D. M., Howard, B. V., and Bennett, P. H. (1979) J. Biol. Chem. 254, 8762–8767
44. Marquardt, H., and Todaro, G. J. (1982) J. Biol. Chem. 257, 5220–5225
45. Cooper, J. A., Bowen-Pope, D. F., Raines, E. W., Ross, R., and Hunter, T. (1982) Cell 31, 263–273
46. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1982) Science (Wash. D. C.) 215, 186–187
47. Kasuga, M., Zick, Y., Blithe, D. L., Crettz, M., and Kahn, C. R. (1982) Nature (Lond.) 298, 667–669
48. Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Häring, H. U., and Kahn, C. R. (1982) J. Biol. Chem. 257, 9891–9894
49. Nishimura, J., and Deuel, T. F. (1981) Biochem. Biophys. Res. Commun. 103, 355–361
Characterization of platelet-derived growth factor-stimulated phosphorylation in cell membranes.

L J Pike, D F Bowen-Pope, R Ross and E G Krebs

J. Biol. Chem. 1983, 258:9383-9390.

Access the most updated version of this article at http://www.jbc.org/content/258/15/9383

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/15/9383.full.html#ref-list-1