Characterization of Platelet-derived Growth Factor α Receptor Synthesis and Metabolic Turnover

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Abstract

Cellular responses to the AA isoform of platelet-derived growth factor (PDGF-AA) are mediated via the PDGF α receptor. Several studies suggest this receptor may signal pathways distinct from those activated by the PDGF β receptor. Because α receptors are less well characterized than are β receptors, and because the quantity of cell surface PDGF receptors governs the extent and perhaps type of PDGF-stimulated response, we examined the synthesis and degradation of α receptors in BALB/c-3T3 cells. Our data show that the ligand-independent half-life of α receptors is 3 h and that optimal turnover of α receptors requires protein synthesis. In the presence of ligand, the half-life of α receptors markedly decreases and is independent of protein synthesis. Although PDGF-AA accelerated the rate of α receptor turnover, pretreatment of cells with PDGF-AA and essentially complete down-regulation of α receptors did not correspondingly increase the level of α receptor synthesis. These findings indicate that the number of cell surface PDGF α receptors is regulated by the rate of internalization of these receptors. Lastly, we report that the recovery of PDGF-AA binding following down-regulation of α receptors is not affected by inhibition of RNA synthesis. Thus, repopulation of cell surface PDGF α receptors may not necessitate an increase in the level of PDGF α receptor mRNA.

Introduction

Two types of receptors for PDGF, termed α and β, have been described. The BB isoform of PDGF (PDGF-BB) interacts with both receptor types, whereas the AA isoform (PDGF-AA) only recognizes α receptors. In comparison to β receptors, which have been well characterized, much less is known about the more recently identified α receptors (1, 2).

It was originally thought that α and β receptors, both of which are ligand-activated tyrosine kinases, were coupled to the same second messenger systems and functioned in an analogous manner (3). While PDGF-BB and PDGF-AA activate many of the same pathways (4, 5), recent studies suggest that the biological activities of these isoforms are not identical. For example, PDGF-BB has been shown to stimulate cellular chemotaxis, membrane ruffling, and cytoskeletal reorganization in human fibroblasts, whereas PDGF-AA does not (6–9). We have found that although both isoforms activate the serum response element in mouse fibroblasts, activation by PDGF-AA but not PDGF-BB is protein kinase C dependent (10). Further evidence suggesting that α receptors induce responses via mechanisms distinct from those of β receptors (or a combination of β and α receptors) is our observation that quiescent BALB/c-3T3 cells require a longer exposure period to PDGF-AA than to PDGF-BB to induce a comparable percentage of the population to initiate DNA synthesis (11).

Differences in the substrate specificities of PDGF α and β receptors have also been described. Although both receptors, independently expressed in receptor-negative cells, tyrosine-phosphorylated phospholipase C-γ, and phosphatidylinositol-3-kinase with similar stoichiometry, α receptors displayed markedly less affinity for a third substrate, GTPase activating protein, than did β receptors (12). It is also noted that PDGF α and β receptors differ in their temporal expression during development. In the developing mouse, α receptors, as well as PDGF-AA, are expressed earlier than are β receptors (13). Palmieri et al. (14) showed that the α receptor is functional i.e., possesses ligand-activatable tyrosine kinase activity, at the mouse blastocyst (day 3.5 preimplantation) and postimplantation (day 7.5) stages, thus suggesting that α receptors may mediate specific events in early development.

Given the capacity of PDGF-BB and PDGF-AA to stimulate distinct cellular events, and because the magnitude of PDGF-mediated signal transduction events is governed by the quantity of cell surface receptors, processes regulating the synthesis and degradation of α and β receptors are important determinants of both the type and level of PDGF-stimulated response. Both α and β receptors are synthesized as Mr 120,000 precursor proteins that are posttranslationally modified to produce mature glycosylated membrane-associated receptors of Mr 170,000–180,000 (15–18). When bound to ligand, both receptors are rapidly internalized and degraded; PDGF receptors are not recycled; thus, repopulation of cell surface receptors following PDGF-induced “down-regulation” is dependent on protein synthesis (16, 19).

PDGF α receptors may participate in unique cellular responses and at specific times in development. Since the regulation of the expression of these receptors has not as yet been described, we examined α receptor synthesis and degradation in the mouse fibroblast cell line, BALB/c-3T3. Our data show that the rate of turnover of PDGF α receptors is regulated by both protein synthesis and PDGF-AA, whereas the rate of synthesis of α receptors is not affected by either PDGF-AA or inhibition of RNA synthesis.
Results

Half-Life of PDGF α Receptors. To determine the ligand-independent half-life of cell surface PDGF α receptors, we used a well documented technique involving specific binding of iodinated ligand to cells treated with cycloheximide for various lengths of time. Because cycloheximide, an inhibitor of protein synthesis, prevents the replacement of internalized receptors with newly synthesized receptors, this assay measures the time required for loss of specific binding and thus provides an estimate of receptor half-life. Cycloheximide was added to density-arrested BALB/c-3T3 cells in spent medium. After incubation at the indicated times at 37°C (Fig. 1), cells received 125I-PDGF-AA for 3 h at 4°C, and specific binding was determined as described in "Materials and Methods." As illustrated in Fig. 1, the half-life of cell surface-expressed PDGF α receptors, as assessed by binding analysis of cycloheximide-treated cells, was 8 h.

Because previous studies had shown that the half-life of the β receptor was only 1-3 h (15-17), and because the half-life of α receptors as determined above considerably exceeds the rate of recovery of PDGF-AA binding (50% increase in 2-3 h; Ref. 20, Fig. 5), we thought the half-life approximated by binding analysis of cycloheximide-treated cells might be artifactual due to the abrogation of protein synthesis. We therefore evaluated the rate of PDGF α receptor turnover by a "pulse/chase" protocol previously used to approximate PDGF β receptor half-life (15-17). Density-arrested BALB/c-3T3 cells were prelabeled with [35S]methionine and subsequently "chased" for various times at 37°C with medium containing unlabeled methionine. PDGF α receptors were immunoprecipitated from cell lysates using specific antiserum, and immunoprecipitated material was analyzed by SDS-PAGE and autoradiography. It is noted that this protocol does not distinguish surface-localized receptors and internalized receptors; thus, loss of radiolabeled receptor presumably reflects receptor degradation.

Using this methodology, we found that the level of PDGF α receptors was reduced by 50% within 3 h (Fig. 2). Furthermore, as also shown in Fig. 2, addition of cycloheximide to the chase medium increased α receptor half-life to 6 h, a time similar to that obtained in binding assays (Fig. 1). Taken together, the data in Figs. 1 and 2 show that the half-life of the PDGF α receptor is 3 h and that inhibition of protein synthesis reduces the ligand-independent rate of internalization and degradation of α receptors. We have found that the half-life of the PDGF β receptor is also increased in the absence of protein synthesis (data not shown). These data suggest that the use of cycloheximide to evaluate receptor half-life may result in anomalous estimates.

Ligand-dependent Down-Regulation of PDGF α Receptors. The rate at which cell surface-localized receptors are internalized is increased in the presence of ligand through a process termed receptor down-regulation. Because inhi-
bition of protein synthesis extended the half-life of PDGF α receptors in the absence of ligand, we examined the effect of cycloheximide on ligand-dependent α receptor down-regulation. Cells prelabeled with [35S]methionine were exposed to PDGF-AA (50 ng/ml) with or without cycloheximide for various lengths of time, and PDGF α receptors were immunoprecipitated from cell lysates. The data in Fig. 3A demonstrate that the half-life of radiolabeled PDGF α receptors chased in medium containing 50 ng/ml PDGF-AA and unlabeled methionine was approximately 10 min (determined by densitometric analysis) and, for the most part, was not affected by cycloheximide. Slightly more labeled α receptor was present in the PDGF-AA (as compared to the PDGF-AA plus cycloheximide) sample at 10 min; this may reflect the incorporation of residual [35S]methionine into protein in the absence but not presence of cycloheximide.

The effect of cycloheximide on the concentration of ligand required for down-regulation of α receptors was also determined. Density-arrested BALB/c-3T3 cells prelabeled with [35S]methionine were incubated for 1 h at 37°C with various concentrations of PDGF-AA in the presence or absence of cycloheximide. PDGF α receptors were immunoprecipitated from cell lysates and electrophoresed on SDS-polyacrylamide gels. The data in Fig. 3B show that the dose-dependent disappearance of α receptors was not altered by cycloheximide; with and without protein synthesis, essentially complete loss of radiolabeled receptor occurred at ≥29 ng/ml PDGF-AA. Thus, while cycloheximide significantly extended the ligand-independent half-life of PDGF α receptors, it did not affect PDGF-AA-mediated α receptor down-regulation. Results similar to those shown in Fig. 3 were obtained in experiments in which α receptors were down-regulated with PDGF-BB (data not shown).

**Recovery of PDGF α Receptors following PDGF-AA Treatment.** Because PDGF α receptors are not recycled following their down-regulation, the time required for repopulation of cell surface α receptors is directly related to the rate at which α receptors are synthesized and transported to the cell surface. As PDGF-AA accelerates α receptor internalization, we asked whether it also enhanced α receptor synthesis. The capacity of epidermal growth factor to up-regulate the synthesis of its receptors has been described previously (21–23). To assess the effect of ligand on α receptor synthesis, BALB/c-3T3 cells were pretreated with or without 50 ng/ml PDGF-AA for 1 h at 37°C. Cultures then received medium containing [35S]methionine but lacking PDGF-AA and were harvested at various times after refeeding. The level of newly synthesized α receptor was determined by immunoprecipitation. As shown in Fig. 4, the incorporation of [35S]methionine into the mature form of the PDGF α receptor progressively and comparably increased in control and PDGF-AA-pretreated cells. PDGF α receptor synthesis in PDGF-AA-pretreated cells was not affected by suramin, an anionic compound that prevents PDGF/PDGF receptor interaction and thus precludes potential α receptor internalization by any residual PDGF-AA (data not shown). The immature form of the PDGF α receptor does not accumulate, and thus the time-dependent decrease in the amount of immature receptor seen in Fig. 4 presumably reflects its progressively lower percentage of total labeled protein in normalized cell extracts.

These data demonstrate that exposure of quiescent BALB/c-3T3 cells to PDGF-AA and consequent down-regulation of PDGF α receptors does not increase the rate of PDGF α receptor synthesis. Thus, the recovery of PDGF-AA binding following down-regulation of α receptors is dependent upon the normal rate of receptor synthesis and processing occurring in the absence of ligand.

**RNA Synthesis Is Not Required for PDGF α Recovery.** The repopulation of PDGF α receptors at the cell surface following their down-regulation is dependent on protein synthesis. To determine if mRNA synthesis was also required, the following experiment was done. Confluent BALB/c-3T3 cultures were pretreated with 50 ng/ml of PDGF-AA for 1 h at 37°C. Cells were then incubated for various times at 37°C in medium containing unlabeled methionine and either PDGF-AA (50 ng/ml) or PDGF-AA plus cycloheximide (Chx; 10 µg/ml). PDGF α receptors were immunoprecipitated and analyzed as described in the Materials and Methods. The position of the 200-kilodalton molecular mass protein marker is indicated. Then, the presence or absence of cycloheximide (10 µg/ml) was determined by densitometric analysis and, for the most part, was not affected by cycloheximide. Slightly more labeled α receptor was present in the PDGF-AA (as compared to the PDGF-AA plus cycloheximide) sample at 10 min; this may reflect the incorporation of residual [35S]methionine into protein in the absence but not presence of cycloheximide.
PDGF-AA for 1 h at 37°C; cells were then assayed immediately or incubated for an additional 2 h at 37°C in medium containing either no addition, cycloheximide, or the RNA polymerase II inhibitor DRB (24). The concentration of DRB used in these experiments inhibited greater than 99% of [3H]uridine incorporation into poly(A)-selected hnRNA. 

Cell surface-localized PDGF α receptor was quantitated by binding assays with 125I-PDGF-AA at 4°C. As shown in Fig. 5, 125I-PDGF-AA binding, which was virtually abolished by a 1-h exposure of cells to PDGF-AA, recovered approximately 60% within 2 h of removal of PDGF-AA. The level of PDGF-AA binding activity was comparable at 2 h in control cells and in cells receiving DRB, thus indicating that ongoing mRNA synthesis may not be needed for restoration of PDGF-AA binding. As expected, PDGF-AA binding did not increase in cells pretreated with PDGF-AA and subsequently exposed to cycloheximide.

Discussion
We have examined the synthesis and degradation of PDGF α receptors in BALB/c-3T3 mouse fibroblasts. Our data show that the ligand-independent half-life of PDGF α receptors, as determined by immunoprecipitation of metabolically labeled α receptors, is approximately 3 h. This half-life is similar to that previously reported for PDGF β receptors (15–17). We also find that the half-life of both α and β receptors increases to approximately 8 h in the absence of protein synthesis (Fig. 1 and 2 and data not shown). In studies involving cycloheximide, receptor half-life was assessed both by immunoprecipitation of radiolabeled receptors (Fig. 2) and by binding analysis of intact cells using 125I-PDGF-AA (Fig. 1). The capacity of cycloheximide to extend PDGF receptor half-life indicates that the rate of PDGF receptor turnover in the absence of ligand is augmented by an as yet undetermined protein synthesis-dependent process. It is possible that protein synthesis may be required for the ligand-independent turnover of other types of cell surface receptors as well. Therefore, to obtain accurate measurements of receptor half-life, receptor internalization kinetics should be determined in the absence of protein synthesis inhibitors.

Previous studies have shown that the half-life of the PDGF β receptor decreases from approximately 2 h to approximately 30 min following exposure of cells to PDGF-BB (15–17). Similarly, we find that PDGF-AA reduces the half-life of PDGF α receptors from 3 h to 10–20 min. In contrast to ligand-independent half-life, the ligand-dependent half-life of PDGF α receptors was not affected by cycloheximide. Both the time-dependent (Fig. 3A) and dose-dependent (Fig. 3B) down-regulation of PDGF α receptors were comparable in cultures receiving PDGF-AA either alone or with cycloheximide. The ligand-dependent half-life of PDGF β receptors was also unaffected by inhibition of protein synthesis (data not shown). Thus, internalization/degradation of PDGF receptors presumably occurs by two distinct mechanisms; in the absence of ligand, optimal turnover of receptors requires protein synthesis, whereas in the presence of ligand, the protein synthesis-dependent process is superseded.

We have also found that although treatment of BALB/c-3T3 cells with PDGF-AA increases the rate of α receptor

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Fig. 4. Level of synthesis of PDGF α receptors. Confluent BALB/c-3T3 cultures were incubated in the presence (Q) or absence (Q) of 50 ng/ml PDGF-AA for 1 h at 37°C. Cultures were then rinsed and refed with medium containing [3H]methionine but lacking PDGF-AA; cells were incubated at 37°C and harvested at the specified times for determination of PDGF α receptor protein by immunoprecipitation. The positions of the 200-kilodalton molecular mass protein marker and of the mature and immature forms of the PDGF α receptor are indicated.

Fig. 5. Recovery of PDGF α receptors in the absence of RNA synthesis. Density-arrested BALB/c-3T3 cells received medium alone or medium supplemented with 50 ng/ml PDGF-AA for 1 h at 37°C. Untreated (Q) and PDGF-AA-treated (0) cultures were harvested at this time. Some PDGF-AA-pretreated cultures were incubated an additional 2 h at 37°C in fresh medium containing either no addition (2), 100 μM DRB (2+DRB), or 1 μg/ml cycloheximide (2+Chx). 125I-PDGF-AA binding was assayed at 4°C as described in "Materials and Methods." 125I-PDGF-AA binding is expressed as the percentage of untreated control (Q) cells.

* J. E. Olson and W. J. Pledger, unpublished data.
turnover, it does not affect the overall level of α receptor synthesis. The amount of α receptor synthesis, as determined by continuous labeling of PDGF-AA-pretreated cells for up to 2 h with [35S]methionine, was comparable in control and treated cultures (Fig. 4). Results from this experiment indicate that the immature form of the PDGF α receptor is fully processed to mature receptor by 1 h. Previous studies on human fibroblasts also found that the processing of immature to mature PDGF α receptor in the absence of PDGF-AA required 1 h (25). As the amount of PDGF-AA presented to cells during the pretreatment period was sufficient to down-regulate all detectable PDGF α receptors, our data show that neither PDGF-AA nor essentially complete loss of α receptors accelerates recovery of cell surface α receptors. Lastly, the capacity of PDGF-AA to affect the turnover but not the synthesis of α receptors indicates that the number of cell surface α receptors is determined by the rate of internalization and degradation of these receptors.

In our findings, Eriksson et al. (26) reported that continuous exposure of human fibroblasts to PDGF-AA did not up-regulate PDGF-AA binding activity. Although the effect of PDGF-AA on the synthesis of α receptors was not examined, these authors (26) showed that PDGF-BB, when present on cells for at least 2 h, increased the synthesis of the α receptor precursor approximately 2-fold. In our system, however, we found that production of the mature form of the α receptor was not significantly enhanced by PDGF-BB pretreatment (data not shown).

A variety of agents have been shown to modulate the level of the PDGF α receptor transcript. Such agents include PDGF-BB (26) and retinoic acid (27), which increased α receptor mRNA in embryonal carcinoma cells and human fibroblasts, respectively, and transforming growth factor β (28) and serum (2), which decreased α receptor mRNA in specific systems. In contrast to PDGF-BB, PDGF-AA did not increase α receptor mRNA levels in human fibroblasts (26).

As shown here, the recovery of PDGF α receptors following their down-regulation by PDGF-AA was not dependent on ongoing RNA synthesis. Similar increases in 125I-PDGF-AA binding were observed in PDGF-AA-pretreated cells incubated in the presence and absence of the mRNA synthesis inhibitor DRB for times up to 6 h (Fig. 5 and data not shown). The rate of synthesis of α receptors was also unaffected by DRB (data not shown). These data indicate that the α receptor message appears to be relatively stable and that repopulation of cell surface α receptors may not necessitate an increase in the level of α receptor mRNA, regardless of whether cells are pretreated with PDGF-AA or PDGF-BB.

Materials and Methods

Cell Culture. Stock cultures of BALB/c-3T3 mouse fibroblasts (clone A31) were grown in Dulbecco-Vogt modified Eagle’s medium supplemented with 10% calf serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in humidified CO2/95% air. For experimental procedures, cells were grown to confluency and used 2–3 days after growth cessation.

PDGF Binding Assays. Recombinant PDGF-AA was labeled with 125I (cot, Boston, MA) using iodobeads (29). For PDGF-AA binding studies, cells in 35-mm culture dishes were placed on ice, rinsed twice with ice-cold binding buffer (Hanks’ buffered saline solution, pH 7.4, supplemented with 0.25% bovine serum albumin), and incubated for 1 h at 4°C in binding buffer containing 3 ng/ml 125I-PDGF-AA. Cells were solubilized with 1% Triton-X 100/0.1% bovine serum albumin. The amount of cell-bound radioactivity was determined using a gamma counter and was corrected for nonspecific binding occurring in the presence of 200 ng/ml unlabeled PDGF-AA. The concentrations of cycloheximide and DRB used blocked greater than 99% protein and RNA synthesis, respectively.

Immunoprecipitation of PDGF Receptors. Cells were labeled with [35S]methionine (100 μCi/ml; NEN; specific activity, >800 μCi/ml) in methionine-free medium containing 0.1% platelet-poor plasma for the times indicated in the figure legends. For chase experiments, cells were then placed in medium containing the standard amount of methionine and specified additions. Cultures were rinsed with phosphate-buffered saline and lysed with a Triton-glycerol buffer (1% Triton-X 100, 10% glycerol, 1 μM phenylmethylsulfonyl fluoride, 100 μM sodium vanadate, 1 mM NaF and 20 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2). Samples were normalized for trichloroacetic acid-precipitable cpn and precleared with Pansorbin (CalBiochem, San Diego, CA) for 2 h at 4°C. PDGF α receptors were immunoprecipitated as described previously (20). Immunoprecipitated material was analyzed on 7.5% SDS-polyacrylamide gels. The relative intensities of the α receptor bands in autoradiograms were quantitated using an LKB laser densitometer.

Antibodies. Antiserum specific for the carboxy terminal (amino acids 980–1089) of the mouse PDGF α receptor was made as described previously (20). PDGF α receptor antiserum was also generously provided by Drs. Dan Bowen-Pope and Ron Seifert (University of Washington, Seattle, WA).

Materials. Recombinant PDGF-AA (>95% pure by SDS-PAGE) and PDGF-BB (>95% pure by SDS-PAGE) were obtained from Biosource, Inc. (Westlake, CA). All other materials were of reagent grade quality and were purchased from the appropriate vendor. For all of the data presented, each set of experiments was repeated a minimum of three times.

Acknowledgments

We thank John Neff for excellent technical assistance.

References


