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BIOSYNTHESIS AND PROCESSING OF THE PLATELET DERIVED GROWTH FACTOR TYPE α RECEPTOR

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SUMMARY: The homodimers (AA, BB) of the platelet derived growth factor (PDGF) differentially interact with two highly related PDGF receptors (α, β) that appear to mediate different functional responses in different cell types. To seek a basis for these apparent functional differences, we investigated the processing of the PDGF α-receptor. The PDGF α-receptor is rapidly glycosylated to a 160 kD form and undergoes a number of intermediate glycosylation steps that result in a mature form of 185 kD that appears at the cell surface within 60-90 minutes. The α receptor has a half-life of ~4 1/2 hours without and ~20 minutes in the presence of ligand. The processing steps of the α-receptor are similar to the processing of the PDGF β receptor, suggesting that differential binding of signalling molecules to activated receptors may be responsible for the apparent functional differences in cellular responses to PDGF AA and PDGF BB.

The platelet-derived growth factor (PDGF) is a potent mitogen and chemoattractant for cells of mesenchymal origin (1). Three different isoforms of PDGF have been described, including a heterodimer of A- and B-chains (PDGF AB) and the homodimers of the A- and B-chains (PDGF AA, PDGF BB) (1-3). The gene encoding the B-chain of PDGF, the normal cellular counterpart of the transforming gene (v-sis) of the simian sarcoma virus (SSV), is transforming when it is highly expressed in NIH 3T3 cells (4,5) whereas the PDGF A-chain gene is not transforming when expressed at equally high levels (6-8) in the same cells. PDGF BB binds to each of the two PDGF receptors (α and β) while PDGF AA binds with high affinity only to the type α receptor (9). The predicted amino acid sequences of the α and β PDGF receptors establish strikingly similar structural domains (8,10-12). However, although the α and β receptors mediate many of the same cellular responses in

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target cells, significant differences appear to exist as well (13-15). The most important difference perhaps is that when cells overexpress v-sis, but not when cells overexpress PDGF A, both the α and β PDGF receptors are activated within the cellular processing compartments prior to expression at the cell surface and that this difference may be related to the transforming potential of v-sis (5).

In order to initiate efforts to dissect differences in the responses of cells to PDGF AA and BB, it is necesary to more fully characterize the receptors themselves; the processing of the type β receptor has been studied in detail (16-18). We now report results of experiments that establish the major processing events of the PDGF α receptor.

MATERIALS AND METHODS

^{35}S-labeling of cells and immunoprecipitation. NIH 3T3 cells (2 x 10^6 cells) were seeded in 100 mm dishes and grown to 90-100% confluence in 2-4 days. The culture media were then replaced with DMEM minus methionine supplemented with Trans^{35}S-label (ICN) at 150 μCi/ml, incubated for 20 minutes at 37°C, washed twice with phosphate buffered saline, and chased in DMEM containing a 1000-fold molar excess of unlabeled methionine. Cells were harvested and lysed as described (8). The lysates were pooled, incubated for 20 min. at 0°C, and centrifuged at 13,000xg for 5 min. Aliquots of the supernatants were incubated with 10 μl of unfractionated antisera in lysis buffer to a final volume of 1 ml for a minimum of 1 hour at 4°C, before addition of 10-20 μl of protein A-sepharose CL4B (Sigma) and incubation for 30 minutes. After centrifugation at 13,000 xg for 10 sec., the pellets were washed 5 times with lysis buffer, boiled in loading buffer (36) with 0.5% dithiothreitol, and electrophoresed in 7.5% SDS polyacrylamide gels (SDS-PAGE) (36). The gels were stained with 0.25% Coomassie brilliant blue G-250 in 30% methanol, 7% acetic acid, before drying and autoradiography.

Antisera. The anti-human type α receptor antisera was obtained after repeated intradermal injections of peptides (residues 1056-1072 of the human PDGF α receptor, 11) conjugated to thyroglobulin (37). Two rabbits, #363 and #263, were immunized and adequate titers to the PDGF type α receptor were obtained in each. Only the sera from rabbit #263 was used in these experiments although antisera #262 gave similar results. Optimum antisera concentrations were determined by serial dilutions for each experiment. Specificity of the antisera for the sequences used in immunization was established by including the cognate peptide in incubation to compete with the receptor for antibody (see Figure 1). Anti-type β receptor antibody was produced against a peptide (amino acid residues 958-977, 10) as described above.

Reagents. Two inhibitors of glycosylation, swainsonine (1 μg/ml) and tunicamycin (2 μg/ml) (Sigma), were added to media of cultured cells 2 hours before the addition of label and were present during labeling and chase.

Endoglycosidases H (0.025 ph U/ml) D, and F (25 μU/ml) were used according to Huang and Huang (38). Briefly, immunoprecipitates were heated to 90°C for 5 min. and cooled to room temperature. Aliquots of 10 μl were removed and diluted to 40 μl with 0.15 M Na citrate, phosphate buffer (pH 8.0). Aprotinin was added to a final concentration of 20 μg/ml. The enzyme and toluene (2 μl) were added and the mixture incubated for 16-18 hours at 37°C before adding an equal volume of loading buffer (see above) and analysis by SDS-PAGE.

Trypsin and soybean trypsin inhibitor (Sigma) were used in PBS at 0.1 mg and 0.3 mg/ml, respectively. Cells that had been labeled for 20 minutes and then chased for the appropriate time were washed twice in PBS before the addition of either trypsin alone or trypsin with soybean trypsin inhibitor. Digestions were performed either at 37°C for 10 minutes (data not shown) or at 4°C for 2 hours. Identical results were achieved with both methods. Cell lysates were prepared and immunoprecipitated as described above, except that soybean trypsin inhibitor (0.3 mg/ml) was included in the lysis and wash buffers.

RESULTS

We first analyzed lysates from ^{35}S-methionine pulse-labeled (20 min.) cells chased for the indicated times with unlabelled methionine by immunoprecipitation with anti-α-receptor (263) antisera or with anti-β receptor (control) antisera. In initial experiments, lysates of NIH 3T3 cells labeled for 20 min. and chased for 45 minutes, were immunoprecipitated with anti-α-receptor antisera (263), separated in SDS gels, and analyzed by autoradiography (Fig. 1). As previously reported (11,19), two proteins that migrate at 185 and 160 kDa were observed (Fig. 1, lane 1); these correspond to the mature form of the α receptor and its precursor. Both the 185 and 160 kDa proteins were competitively displaced by the synthetic α receptor peptide during immunoprecipitation but not with the β receptor peptides that were used to raise the respective antisera (Fig. 1, lanes 2-4). Neither protein was immunoprecipitated with pre-immune serum (Fig. 1, lane 5). In other control experiments (data not shown), γ-^{32}P-ATP kinase assays of membrane preparations that were stimulated either with recombinant PDGF AA or BB were performed (20). Both PDGF AA- and BB stimulated phosphorylation of a 185 kDa protein that was immunoprecipitated specifically with anti-α antisera (263). Anti-PDGF β antisera but not anti-α antisera (263) immunoprecipitated a protein of 180 kDa that migrated identically with the previously described type β receptor (18,20). These premilinary experiments thus establish the specificity of antisera 263 for the type α-PDGF receptor and its precursor form and that antisera 263 fails to cross-react with the closely related β-PDGF receptor.

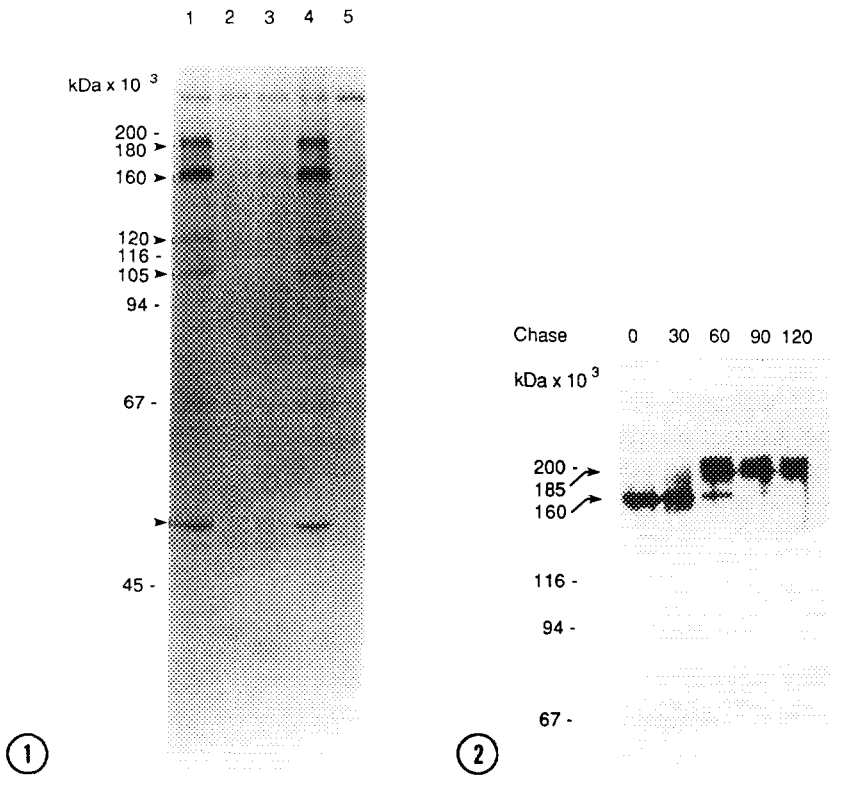


Figure 1. Immunoprecipitation of PDGF type α receptor by antiserum 263. NIH 3T3 cells were pulse labeled and chased for 45 min. to allow for the formation of both the 160 and 185 kDa forms of the receptor. Cell lysates were immunoprecipitated with 1) antiserum 263 alone, 2) antiserum 263 with ~1 μg of cognate peptide conjugated to thyroglobulin, 3) antiserum 263 with ~1 μg of cognate peptide conjugated to poly L lysine, 4) antiserum 263 with ~ 1 μg of a peptide identical in sequence to a protion of the PDGF type β receptor and conjugated to thyroglobulin, and 5) preimmune serum from the rabbit used for the creation of antiserum 263.

Figure 2. The 160 kDa protein is the precursor for the phosphorylated 180 kDa mature receptor. Cells were pulsed with Trans^{35}S-label for 20 min. before being chased in medium containing a 1000-fold excess of unlabeled methionine.

Antisera 263 was then used to analyse the processing steps of the PDGF α receptor in NIH 3T3 cells that were labeled for 20 min with ^{35}S-trans label and chased for the indicated times with unlabeled methionine. The predicted 120 kDa nascent form (11,19) of the PDGF α-receptor was not visualized in repeated experiments even with chase times as short as 5 minutes. The first detectable form of the α receptor migrates at 160 kDa; the 160 kDa form is chased into the mature 185 kDa protein at 60 min (Fig. 2, arrow) and chased completely into the 185 kDa form at 90 min. (Fig. 2, arrows). The results

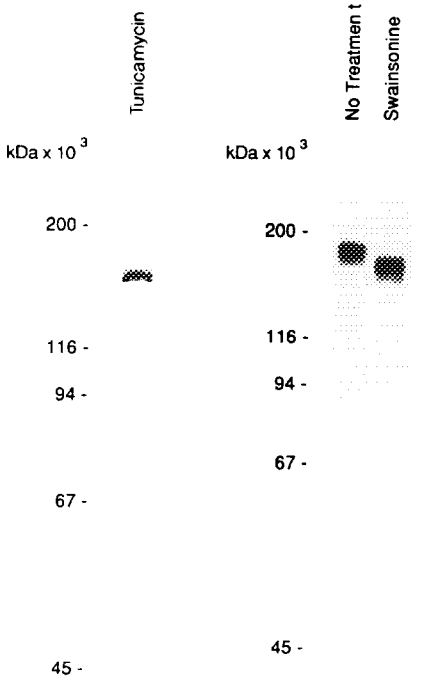
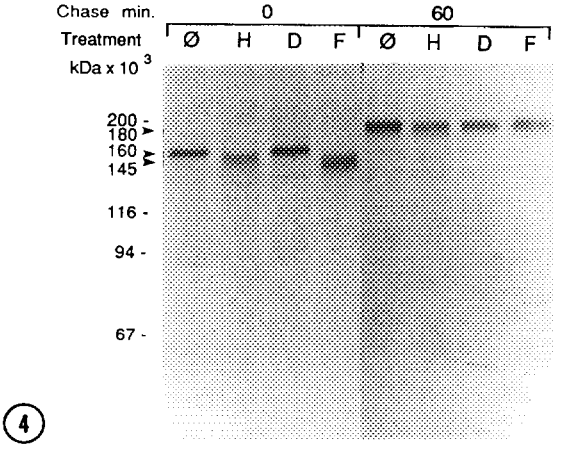


Figure 3. Effect of tunicamycin and swainsonine on the formation of the 160 and 180 kDa receptor. Cells were treated with either tunicamycin or with swainsonine as described (20). Briefly, cells were treated 2 hours before the addition of label with either inhibitor. The same concentrations of inhibitor was maintained during labeling. Cells were harvested 2 hours after initiation of labeling and lysates were immediately immunoprecipitated.

suggest that the nascent 120 kD form is rapidly processed into a 160 kD form that is subsequently and more slowly processed into the 185 kDa mature form of the receptor at 90 min.

In order to investigate possible roles of glycosylation in the processing of the PDGF α receptor, tunicamycin, which inhibits the transfer of N-acetyl glucosamine-1-phosphate to the dolichol phosphate that is required for the addition of glycosides to peptide asparagine residues (21,22), was used. Swainsonine, which inhibits mannosidase II, an enzyme within the Golgi stack that removes two mannose residues from N-linked oligosaccharides, a step required for the subsequent maturation of simple mannose containing carbohydrate adducts to more complex forms (23,24), also was used. In lysates from cells treated with tunicamycin (1μg/ml), a sharp reduction in the intensity of the 160 kDa band (Fig. 3) was associated with the appearance of an intensely labelled band of 145 kDa, suggesting that processing of the α-receptor is blocked at 145 kDa. In immunoprecipitates from swainsonine (1 μg/ml) treated cells, the intensity of the 160 kDa precursor band remained essentially unchanged, but a unique band was observed at 170 kDa (Fig. 3),



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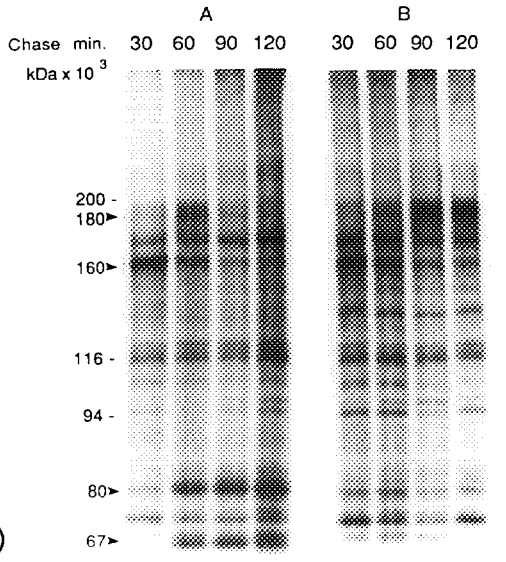

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Figure 4. Endoglycosidase digestion of the 160 and 180 kDa receptor forms. Cells were labeled for 20 min. and either not chased or chased for 60 min. Lanes marked Ø were not treated with glycosidase; those marked H, D, and F were digested with endoglycosidase H, D, or F, respectively.

Figure 5. Presence of the 180 kDa mature form at the cell surface. Cells were pulse/chased as described for Figure 1. One set of dishes was treated with trypsin (A) while the other was treated with trypsin and soybean trypsin inhibitor (B).

suggesting that the 160 kDa intermediate was converted to a 170 kD intermediate which, in the presence of swainsonine, is not further processed to the mature, 185 kD protein. In support of these results, lysates of NIH 3T3 cells were treated with endoglycosidases H, D, and F (Fig. 4). Endoglycosidase H cleaves the chitobiose core of high mannose and certain hybrid oligosaccharides (25,26), endoglycosidase F cleaves glycosidic bonds of the N,N diacetylchitobiose core of many high mannose and biantennary N-linked oligosaccharides (27), and endoglycosidase D cleaves di-N-acetylchitobiose linkage in N-linked oligosaccharides (28). Endoglycosidases H and F converted the 160 kDa form to a protein of ~145 kD, a form of the α receptor that migrates identically to the form that was immunoprecipitated from tunicamycin-treated cells (Fig. 4; lanes H, D, F). In contrast, the 185 kDa mature form of the α receptor (Fig. 4, lane 0) was resistent to digestion by all endoglycosidases, consistent with the resistance of complex oligosaccharides to enzymatic hydrolysis by endoglycosidases H, D, and F.

Cell surface expression. In order to determine the time required for the appearance of newly synthesized α receptor at the cell surface, labelled cells were chased and treated with trypsin at various times after initiation of the

chase. Immunoprecipitates from these cells were analysed in gels. The *de novo* synthesized mature receptor first appeared at the cell surface ~60 min. after the initiation of the chase. However, not all of the 185 kD form was exposed to trypsin at 60 min. In repeated experiments, trypsin failed to completely digest the 185 kDa form of the α receptor, indicating that maturation to the 185 kDa form of the α receptor occurs within the cell before the receptor reaches the cell surface (Fig. 5A, lane 60, arrows). Ninety minutes after initiation of the chase, all of the *de novo* receptor appears at the cell surface, as indicated by its total susceptibility to trypsin digestion (Fig. 5A, lane 90). In control experiments, digestion of the receptor of the cell surface was not observed during the chase if trypsin and soybean trypsin inhibitor were added together (Fig. 5, part B, lanes 30-120).

Half-life of receptor at the cell surface. The half-life of the α receptor at the cell surface was also analysed (data not shown). The T1/2 was estimated at ~4 1/2 hours. When recombinant PDGF A or B homodimers were added to cells at saturating concentrations (100 ng/ml), the T1/2 was reduced to ~20-30 minutes as estimated by densitometric scanning. In a control experiment, the A-chain homodimers had no effect on the T1/2 of the type β receptor.

Receptor associated proteins. Although the type α receptor is the major protein immunoprecipitated with antisera 263, additional proteins of 120, 105, and 50 kDa were consistently associated with immunoprecipitates. The results suggest that the 120, 105, and 50 kDa proteins are receptor associated proteins and not degraded products of the α-receptor; the identification of the apparent receptor associated proteins has not been pursued further.

DISCUSSION

Although the type α and β PDGF receptors are products of different genes and their expression is independently regulated (10,11,19), the results presented here suggest that the processing of the type α receptor is very similar to that of the type β receptor. The cDNA of the PDGF α receptor predicts an initial protein product of 120 kDa with an ~2.8 kDa signal peptide; the earliest detectable protein product (5 min) is pulse chase analysis is ~160 kD. The initial precursor identified in tunicamycin-treated cells is ~145 kD; this likely reflects the addition of O-linked oligosaccharides within the endoplasmic reticulum and an additional contribution of N-linked oligosaccharides to the 160 kD early form of the PDGF α receptor. The inhibition of formation of the mature form of the receptor by swainsonine

suggests that addition of N-acetyl glucosamine to the receptor is followed by mannose addition and subsequent processing to complex forms of the oligosaccharides. Consistent with this sequence of glycosylation events, the carbohydrate residues of the partially processed forms of the PDGF α receptor were readily removed by endoglycosidases H and F, resulting in a protein of ~145 kD that migrated identically with the α receptor in tunicamycin-treated cells.

Maturation of oligosaccharide side chains occurs in the Golgi apparatus (29), a process which gives rise to the mature, 185 kDa form of the α receptor. This process is extremely rapid, *de novo* synthesized receptors are observed at the cell surface starting at ~60 min. of chase and appear to be fully expressed at the cell surface and accessible to exogenous trypsin by 90 min; only the 185 kDa form was detected at the cell surface. Both PDGF AA and BB homodimers cause rapid degradation of *de novo* type α receptor that occurs with a T1/2 of ~20 minutes and that is complete ~90 minutes after the addition of ligand.

The processing of the type α receptor is similar to the processing of the type β receptor previously reported. Previous work has established that each of these receptors is activated internally by v-sis during processing (5,8,30,31). The similarity in processing of these two receptors is consistent with the equal potential of each receptor to be activated internally during processing by v-sis. However, only v-sis is able to activate the two receptors during their processing. The PDGF α-receptor immunoprecipitates appear to contain associated proteins of 120, 105, and 50 kDa. Although the identity of these proteins is not known, at least one of them (120 kDa) has an identical apparent molecular weight with the GTP-ase activating protein (GAP), which is known to associate with the type β receptor (32,33), and the 105 kDa protein may be the β-subunit of phosphatidylinositol 3-kinase (34,35). The further characterization of the PDGF α receptor associated proteins may provide further insights into mechanisms of its function relative to mechanisms governing the function of the PDGF β receptor and may serve to identify possible different downstream signalling events.

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