Ligand-induced Polyubiquitination of the Platelet-derived Growth Factor \( \beta \)-Receptor*

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We have analyzed the nature of ligand-induced shift to higher molecular weight forms of the \( \beta \)-receptor for platelet-derived growth factor expressed in porcine aortic endothelial cells. The modification of the \( \beta \)-receptor was found to be due to polyubiquitination, as judged by immunoblotting using an anti-ubiquitin antiserum. A mutant \( \beta \)-receptor made kinase negative by a point mutation (K634A mutant) did not undergo ubiquitination in response to ligand stimulation. A mutant in which carboxy-terminal 98 amino acids were deleted (CT98 mutant) and which retained kinase activity was likewise not ubiquitinated. These data suggest that the kinase activity, as well as the carboxy-terminal 98 amino acids, is required for ubiquitination of the \( \beta \)-receptor. Ligand-induced degradation of the receptor-bound ligand, as well as of the receptor itself, was partially impaired in the CT98-receptor-expressing cells, suggesting that the ubiquitination is of importance for efficient degradation of the ligand-receptor complex.

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Platelet-derived growth factor (PDGF)\(^1\) is a connective tissue cell mitogen, which is made up of dimers of disulfide-bonded A and B polypeptide chains (for reviews see Heldin and Westermark (1990) and Raines et al. (1990)). Recent studies have established the presence of two types of PDGF receptors that display differences in ligand binding specificity (Yarden et al., 1986; Matsui et al., 1989; Claesson-Welsh et al., 1989b). Ligand binding induces receptor dimerization; the B-subunit of PDGF binds to both \( \alpha \) and \( \beta \)-receptors with high affinity, whereas the A-subunit only binds to the \( \alpha \)-receptor (Heldin et al., 1989; Bishaye et al., 1989; Seifert et al., 1989; Hammacher et al., 1989). The \( \alpha \) and \( \beta \)-receptors are structurally similar; each has a split intracellular protein-tyrosine kinase domain, which becomes activated after ligand binding. Ligand binding is accompanied by clustering of the receptor (Yarden et al., 1989; Sorkin et al., 1991). Analysis of PDGF receptor purified from PDGF-stimulated Bab/l/c 3T3 cells revealed that ubiquitin was covalently bound to the receptor (Yarden et al., 1986). Ubiquitin is present in eukaryotes and is a highly conserved 76-amino acid residue protein (Goldstein et al., 1975). Ubiquitin conjugation has been shown for a variety of proteins, which are implicated in a number of basic cellular functions. A large body of evidence suggests that ubiquitin has a fundamental role in mediating intracellular protein degradation (for reviews see Rechsteiner (1987), Ciechanover and Schwartz (1989) and Hershko (1991)). The biosynthesis of ubiquitin-protein conjugates involves several steps. First, the ubiquitin-activating enzyme, \( E_1 \), activates the carboxyl terminus of ubiquitin (Ciechanover et al., 1981; Haas and Rose, 1982), and then the ubiquitin molecules are transferred to cysteine residues in a family of small carrier proteins called \( E_2 \)s (Hershko et al., 1985; Pickart and Rose, 1986). Finally, ubiquitin is selectively attached to target proteins with (Hershko et al., 1983) or without (Haas et al., 1988; Pickart and Vella, 1988) the participation of the ubiquitin-protein ligase, \( E_3 \).

In the present study, we report that PDGF-BB stimulation induces polyubiquitination of the PDGF \( \beta \)-receptor. Both the kinase activity and the carboxy-terminal 98 amino acids of the receptor are required for the ligand-induced polyubiquitination. Possible functional implications of the ubiquitination of the receptor are discussed.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—A complementary DNA (cDNA) encoding the full-length human PDGF \( \beta \)-receptor (Claesson-Welsh et al., 1988), which was designated wild-type receptor in this study, was subcloned into pBluescript II SK (Stratagene) by using 5'-EcoRI and 3'HindIII (at nucleotide 3629 according to Claesson-Welsh et al. (1988)) sites of the insert. To obtain the mutant receptor with 98 amino acid residues deleted from the carboxy-terminal of the wild-type receptor (CT98 mutant; Mori et al., 1991), the fragment between BstEII (at 2944) and the HindIII sites of the insert was replaced by a fragment generated by polymerase chain reaction (Saiki et al., 1988) to create a translational stop codon at the appropriate site. Two oligonucleotides were used as primers in polymerase chain reaction with the wild-type receptor cDNA. The 5'-primer consisted of nucleotides 3196-3210 followed by a stop codon and a HindIII site. The reaction product was subjected to ethanol precipitation followed by digestion with BstEII and HindIII. The digested product was subjected to ethanol precipitation followed by digestion with BstEII and HindIII. The digested product was used in ligation with phagemid vector pC196, and the resulting recombinant phagemid was transformed into E. coli. The correct recombinant phagemid was identified by Southern blot analysis with the wild-type receptor cDNA as probe.

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\( ^{2} \) The abbreviations used are: PDGF, platelet-derived growth factor; PAA, porcine aortic endothelial; SDS, sodium dodecyl sulfate; WGA-Sepharose, wheat germ lectin-Sepharose 6MB.
to an alanine residue by site-directed mutagenesis (K634A mutant, see Westermann et al. (1990)) were cloned into the retroviral expression vector pZipNeo (Cepko et al., 1984), as described (Westermann et al., 1990).

**Cell Culture and Transfection**—The porcine aortic endothelial (PAE) cell line (Miyazono et al., 1987), which lacks endogenous PDGF α- and β-receptors but stably transfects with a cDNA encoding the wild-type PDGF β-receptor, was used. Cells were labeled with [35S]methionine and then incubated with PDGF-BB at 37 °C for various time periods. After incubation, the cells were lysed, immunoprecipitated with the β-receptor specific antisera PDGFR-3, and the immunoprecipitates were analyzed by SDS-gel electrophoresis followed by fluorography. As shown in Fig. 1, before stimulation of the cells with PDGF-BB, two components of approximately 160 and 190 kDa were immunoprecipitated by PDGFR-3 (Fig. 1, lane 1). It was previously shown that the 160- and 190-kDa forms are the precursor and the mature PDGF β-receptors, respectively (Claesson-Welsh et al., 1988). After PDGF-BB stimulation, the mature receptor band gradually became blurred, and a dense smearing appeared toward the high molecular weight region of the gel. The smearing reached maximal intensity after 7–10 min of incubation with PDGF-BB (Fig. 1, lanes 2–7) and, thereafter, disappeared within 20 min (lane 8). This ligand-induced smearing of the mature receptor band was also observed when the cells were incubated with PDGF-BB at 4 °C (Fig. 2). At 4 °C, the smearing reached maximal intensity after 2 h (Fig. 2, lanes 2–5) and, thereafter, remained unchanged for up to 8 h of incubation with PDGF-BB (data not shown). When the cells were stimulated with PDGF-BB at 4 °C for 120 min and then transferred to 37 °C, the smearing disappeared within 20 min of incubation at 37 °C (Fig. 2, lanes 6–10).

**PDGF β-Receptor Is Ubiquitinated after Ligand Stimulation**—It was reported by Yarden et al. (1986) that a purified murine PDGF receptor preparation contained ubiquitin. In order to examine if the smearing was due to ubiquitination, an immunoblotting assay using an anti-ubiquitin antisera was performed. The wild-type receptor-expressing cells were incubated with PDGF-BB at 4 °C for different time periods. After incubation, a glycoprotein-enriched fraction of the cells, in 0.005 M sodium phosphate, pH 7.5, containing 2% SDS and 0.002 M 2-mercaptoethanol at 60 °C for 30 min, according to the manufacturer's instructions (Amersham Corp.).

**RESULTS**

In order to analyze the effect of PDGF-BB on the structural characteristics of the PDGF β-receptor, PAE cells, lacking endogenous PDGF α- and β-receptors but stably transfects with a cDNA encoding the wild-type PDGF β-receptor, were used. Cells were labeled with [35S]methionine and then incubated with PDGF-BB at 37 °C for various time periods. After incubation, the cells were lysed, immunoprecipitated with the β-receptor specific antisera PDGFR-3, and the immunoprecipitates were analyzed by SDS-gel electrophoresis followed by fluorography. The relative migration positions of molecular weight standards (myosin, 200,000; phosphorylase b, 92,500) run in parallel are indicated.

**Fig. 1.** PDGF-BB-induced smearing of the wild-type PDGF β-receptor at 37 °C. PAE cells expressing the wild-type receptor were labeled for 3 h with [35S]methionine and then incubated with 100 ng/ml PDGF-BB for 0–20 min at 37 °C. After incubation, the cells were lysed, immunoprecipitated with PDGFR-3, and analyzed by SDS-gel electrophoresis and fluorography.
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FIG. 2. PDGF-BB-induced smearing of the wild-type PDGF β-receptor. A, the wild-type receptor-expressing cells were incubated with 100 ng/ml PDGF-BB at 4 °C for 0-120 min at 4 °C (lanes 1-5). Other cell cultures were first stimulated with PDGF-BB at 4 °C for 120 min and then transferred to 37 °C for a continued 1-20 min of incubation (lanes 6-10). After incubation, the cells were processed for immunoprecipitation with PDGFR-3 followed by SDS-gel electrophoresis and fluorography.

FIG. 3. PDGF-BB-induced ubiquitination of the wild-type PDGF β-receptor. A, the wild-type receptor-expressing cells were incubated with 100 ng/ml PDGF-BB at 4 °C for 0-120 min. After incubation, the cells were lysed, and a glycoprotein-enriched fraction of the cells, collected by adsorption to WGA-Sepharose, was separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. The blot was probed with PDGFR-3, and sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham Corp.). B, after removal of the first probe, as described under “Experimental Procedures,” the same blot was reprobed with the anti-ubiquitin antisemur.

FIG. 4. Effect of inactivation of the receptor kinase on PDGF-BB-induced ubiquitination of the PDGF β-receptor. A, the cells expressing the wild-type (WT) or K634A mutant receptors were incubated with or without 100 ng/ml PDGF-BB at 4 °C for 2 h. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 5. The blot was probed with PDGFR-3. B, after removal of the first probe, the same blot was reprobed with the anti-ubiquitin antisemur.

collected by adsorption to WGA-Sepharose, was separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. First, the blot was probed with the β-receptor specific antisemur PDGFR-3. As shown in Fig. 3A, a 190-kDa band, which most likely corresponded to the mature form of the receptor, was detected in each lane; the 160-kDa precursor form is not seen in this experiment because it does not bind to WGA-Sepharose. The pattern of ligand-induced smearing of the 190-kDa component was similar to that observed in the immunoprecipitation assay (Figs. 2 and 3A, lanes 1-5). The same filter was then stripped and reprobed with the anti-ubiquitin antisemur. As shown in Fig. 3B, no specific band was detected before stimulation of the cells with PDGF-BB (Fig. 3B, lane 1). After PDGF-BB stimulation, a broad band of more than 200 kDa was visualized by the anti-ubiquitin antisemur, and the intensity of the band increased with time up to 120 min (Fig. 3B, lanes 2-5). As judged by the position and the appearance of the broad band, it corresponded to the smearing above the band corresponding to the mature form of the receptor, observed using PDGFR-3. Thus, the immunoblotting assay revealed that the ligand-induced broad band of higher molecular weight than the receptor was constituted by components recognized both by PDGF β-receptor antibodies and ubiquitin antibodies.

An Intact Kinase Is Necessary for Ubiquitination of the Receptor—In order to examine the role of the receptor kinase in the ligand-induced smearing of the PDGF β-receptor, we carried out the immunoblotting assay using cells expressing the K634A mutant β-receptor, which was made kinase-negative by a point mutation (Westermarck et al., 1990). The cells were incubated in the presence or absence of PDGF-BB for 2 h at 4 °C. After incubation, the cells were processed for immunoblotting as described above. When the blot was probed with PDGFR-3, bands of approximately 190 kDa were detected both from cells expressing the wild-type receptor and from cells expressing the K634A mutant receptor (Fig. 4A). PDGF-BB stimulation of the K634A mutant-receptor-expressing cells did not induce smearing of the mature receptor band (Fig. 4A, lane 4), whereas, as expected, the smearing was observed in the wild-type receptor-expressing cells (Fig. 4A, lane 2). When the blot was reprobed with the anti-ubiquitin antisemur, no specific band was detected in the K634A mutant-receptor-expressing cells (Fig. 4B, lanes 3-4), whereas a broad band of more than 200 kDa was detected in the wild-type receptor-expressing cells after PDGF-BB stimulation (Fig. 4B, lane 2). Thus, the inactivation of the receptor kinase abolished the ability of the receptor to undergo ligand-induced ubiquitination.

Carboxy-terminal Truncation of 98 Amino Acids Abolishes Ubiquitination of the Receptor—In a series of immunoprecipitation assays using different mutants of the PDGF β-receptor, we noticed that the ligand-induced smearing of the mature receptor band was not observed in cells expressing a mutant PDGF β-receptor in which the carboxy-terminal 98 amino acids were deleted (CT98 mutant). The CT98 mutant receptor was previously shown to possess full ligand-stimulable kinase activity (Mori et al., 1991). Thus, the immunoblotting assay was carried out using the cells expressing the CT98 mutant receptor, with the wild-type receptor-expressing cells as a control. As shown in Fig. 5A, the mature receptor bands of approximately 190 kDa (wild type, lanes 1-2) and 175 kDa (CT98 mutant, lanes 3-4) were detected using a rabbit antisemur, PDGFR-HL2, raised against the PDGF β-receptor kinase insert sequence (Weima et al., 1990). The ligand-induced smearing of the mature receptor band was observed when the wild-type receptor-expressing cells were analyzed.
FIG. 5. Effect of carboxyl-terminal truncation of 98 amino acids on PDGF-BB-induced ubiquitination of the PDGF β-receptor. A, the cells expressing the wild-type (WT) or CT98 mutant receptors were stimulated with PDGF-BB and processed for immunoblotting as described in the legends to Figs. 3 and 4. The blot was probed with PDGFR-HL2. B, after removal of the first probe, the same blot was reprobed with the anti-ubiquitin antiserum.

(Fig. 5A, lane 2), but this pattern was not seen in samples derived from the CT98 mutant receptor-expressing cells (Fig. 5A, lane 4). Furthermore, when the blot was reprobed with the anti-ubiquitin antiserum, no specific band was detected in the CT98 mutant receptor-expressing cells (Fig. 5B, lanes 3–4), whereas the broad band of more than 200 kDa was detected in the presence of PDGF-BB in the wild-type receptor-expressing cells (Fig. 5B, lane 2). Thus, the truncation of 98 amino acids in the carboxyl terminus abolished the ability of the receptor to undergo ligand-induced ubiquitination.

The CT98 mutant receptor was previously found to become internalized after ligand stimulation with approximately the same efficiency as the wild-type receptor (Mori et al., 1991). In order to assess the effect of the ligand-induced ubiquitination on degradation of the receptor, we carried out a pulse-chase analysis in the presence of PDGF-BB using the cells expressing the wild-type and the CT98 mutant receptors. The cells were metabolically labeled for 3 h and then chased for 30 min to allow the labeled receptors to reach the cell surface. Thereafter, the cells were incubated with PDGF-BB for 0–120 min at 37 °C. Degradation was measured by immunoprecipitation of the total population of labeled receptors from cell lysates at different time points after PDGF-BB addition. As shown in Fig. 6A, the intensity of the mature receptor bands of 190 kDa (wild type, lanes 1–5) and 175 kDa (CT98 mutant, lanes 6–10) was substantially reduced after ligand stimulation. Fig. 6B shows the relative intensity of the mature receptor band at each time point after PDGF-BB addition, measured by densitometric scanning of the fluorogram shown in Fig. 6A. The rate of degradation of the CT98 mutant receptor was slower than that of the wild-type receptor; 2-h exposure to PDGF-BB caused almost complete disappearance of the mature form of the wild-type receptor, whereas 20% of that of the CT98 mutant receptor still remained. The same result was obtained using another PDGF β-receptor antiserum, PDGFR-1, in the immunoprecipitation step (data not shown).

DISCUSSION

The present study shows that the PDGF β-receptor undergoes ubiquitination as an early response to PDGF-BB stimulation. This observation explains why Yarden et al. (1986) found the amino-terminal sequence of ubiquitin in conjunction with their attempts to determine the NH2-terminal amino acid sequence of the PDGF receptor purified from PDGF-stimulated Balb/c 3T3 cells. Several ubiquitin molecules (from 1 to more than 20) have been found to be linked to proteins as ordered chains of branched ubiquitin-ubiquitin conjugates (Chau et al., 1989). Therefore, the apparent increase in the relative molecular mass and the formation of the smearing pattern of the mature form of the PDGF-BB-stimulated receptor that we observed in the present study are most likely due to polyubiquitination of the mature form of the receptor. We cannot completely rule out the possibility that other modifications also participate in the formation of the ligand-induced smearing. However, a series of chemical and enzymatic treatments, including treatments with alkali (0.1 M NaOH, at 37 °C for 1 h), acid (0.1 M HCl, at 37 °C for 1 h), NH4OH (0.2 M, pH 7.5, at 37 °C for 30 min), alkaline phosphatase (Boehringer Mannheim), and phosphodiesterase I (Pharmacia), failed to alter the smearing pattern (data not shown), suggesting that for instance phosphorylation and ADP-ribosylation (Nishizuka et al., 1968) are not the cause of the smearing.

It has been demonstrated that three enzyme components of the ubiquitin-protein ligase system, E1, E2, and E3, are required for ubiquitin ligation (Hershko et al., 1983). Ubiquitin is first activated by a specific activating enzyme (E1) (Chiechanover et al., 1981; Haas and Rose, 1982) and is then transferred to a ubiquitin-carrier protein (E2). The latter is the donor for the ligation of ubiquitin to target proteins, a process carried out by ubiquitin-target protein ligase, E3. E3 appears to have a central role in selection of target proteins (Hershko et al., 1986), and the NH2 terminus of intracellular target proteins is an important structural determinant for their recognition by the ubiquitin ligase system (Reiss et al., 1988).

FIG. 6. PDGF-BB-stimulated degradation of the wild-type and the CT98 mutant PDGF β-receptors. A, the cells expressing the wild-type (WT) or CT98 mutant receptors were labeled for 3 h with [35S]methionine and then chased with an excess of unlabeled methionine for 30 min. Thereafter, the cells were incubated with 100 ng/ml PDGF-BB for 0–120 min at 37 °C. After incubation, the cells were processed for immunoprecipitation as described in the legend to Fig. 1, except that PDGFR-HL2 was used instead of PDGFR-3. B, densitometric scanning of the fluorogram of the mature forms of the wild-type (○) and CT98 mutant (○) receptors. The intensity is expressed as percent of that at time zero. The rate of receptor loss in the absence of ligand (+; data from a fluorogram which is not shown) was not significantly different for the wild-type and CT98 mutant receptors and is shown as the average value for these two receptors.
The mechanism involved in the recognition of the membrane-bound proteins by the ubiquitin ligase system is, however, not known. In the case of the PDGF β-receptor, inactivation of receptor kinase abolished the ligand-induced ubiquitination of the receptor (Fig. 4). Thus, it is possible that receptor autophosphorylation and/or phosphorylation of exogenous substrates are important for its recognition by the ubiquitin ligase system. However, we found that PDGF-BB-induced ubiquitination of a mutant PDGF β-receptor, in which the two major autophosphorylation sites, Tyr-751 and Tyr-857 (Kazlauskas and Cooper, 1989), were simultaneously altered to phenylalanine residues by site-directed mutagenesis, also occurred with equal efficiency as the wild-type receptor (data not shown), suggesting that phosphorylation of these two tyrosine residues is not necessary for the ubiquitination process.

Our data also showed that a carboxyl-terminal deletion of 98 amino acids (CT98 mutant) abolished the PDGF-BB-induced ubiquitination of the truncated PDGF β-receptor (Fig. 5). The CT98 mutant receptor was found to mediate a mitogenic signal and to be autophosphorylated to an extent similar to that of the wild-type receptor (Mori et al., 1991), suggesting a role for the carboxyl terminus in the ubiquitination which is distinct from the kinase function of the receptor. It has been shown that the ubiquitin molecule is linked by an isopeptide linkage to an ε-amino group of a lysine residue of the target protein (Ciechanover et al., 1989). The carboxyl-terminal 98-amino acid stretch contains only 1 lysine residue (Lys-1029 according to the numbering of Claesson-Welsh et al., 1988), and thus it is theoretically possible that this lysine residue is the acceptor for ubiquitination of the PDGF β-receptor. However, a mutant PDGF β-receptor, in which Lys-1029 was changed to an alanine residue by site-directed mutagenesis, was found to be ubiquitinated after PDGF-BB stimulation as efficiently as the wild-type receptor (data not shown). Therefore, the 98-amino acid region or parts thereof may serve as the recognition site for the ubiquitin ligase system but apparently do not contain the acceptor site for the ubiquitin molecules. Alternatively, the region may be necessary for maintenance of a three-dimensional structure of the receptor which is important for the interaction of the receptor with the ubiquitin ligase system.

PDGF-BB stimulation has previously been shown to lead to an increased rate of internalization and degradation of ligand and receptor in lysosomes. In the CT98 mutant receptor-expressing cells, the rate of ligand-induced degradation of the receptor after PDGF-BB stimulation, as assessed by pulse-chase analysis followed by immunoprecipitation, was reduced as compared with the wild-type receptor-expressing cells (Fig. 6). Furthermore, the rate of degradation of receptor-bound ligand in the CT98 mutant receptor-expressing cells was about half that in the wild-type receptor-expressing cells (Mori et al., 1991). These data suggest the possibility that the ligand-induced polyubiquitination of the PDGF β-receptor is involved in the intracellular transport or sorting of the ligand-receptor complex or is of importance for efficient degradation of the complex. An artificial mutant of the epidermal growth factor receptor, which escapes ligand-induced degradation due to removal of an internalization domain, has been shown to transduce an amplified growth response (Chen et al., 1989; Wells et al., 1990). It is thus conceivable that efficient internalization and degradation of the ligand-stimulated PDGF β-receptor, possibly involving receptor ubiquitination, is an important mechanism for control of growth stimulation.

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