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LIGAND-ACTIVATED PLATELET-DERIVED GROWTH FACTOR β-RECEPTOR IS DEGRADED THROUGH PROTEASOME-DEPENDENT PROTEOLYTIC PATHWAY

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The platelet-derived growth factor β-receptor undergoes polyubiquitination as a consequence of ligand binding. Ubiquitin conjugation to protein is implicated in proteasome-dependent proteolytic pathway for short-lived proteins. In the present study, we have examined effects of different kinds of cell-penetrating proteasome inhibitors, including N-benzyloxycarbonyl-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal (PSI) and a *Streptomyces* metabolite lactacystin, on ligand-stimulated degradation of the β-receptor. These proteasome inhibitors were found to considerably inhibit the degradation of autophosphorylated and polyubiquitinated receptors, suggesting the possible involvement of proteasomes in the degradation process of the ligand-activated β-receptor.

Platelet-derived growth factor (PDGF) promotes the growth of mesenchymal cells in normal and pathological processes (1). Two types of the receptor for PDGF, designated α- and β-receptors, have been identified (2-4). They both belong to the receptor tyrosine kinase subfamily III (5), consisting of five immunoglobulin-like repeats in the extracellular part and an intracellular tyrosine kinase domain, which is split into two parts by a non-catalytic insertion sequence, the kinase insert.

We have previously reported that the PDGF β-receptor undergoes polyubiquitination as a consequence of ligand binding (6), and have suggested that the ligand-induced ubiquitination plays a negative regulatory role in mitogenic signaling of the PDGF β-receptor, possibly by promoting the efficient degradation of the ligand-activated receptor (7). Ubiquitin is present in eukaryotes and is a highly conserved 76-amino acid residue protein (8). Evidence supports the concept that ubiquitin conjugation

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The abbreviations used are: PDGF, platelet-derived growth factor; PAE, porcine aortic endothelial; WGA, wheat germ lectin.

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to protein is implicated in ATP-dependent proteolytic pathways for short-lived proteins such as cyclins, Myc, Fos, and p53 (see Ref. 9, for a review). A multisubunit 26S (> 2000 kDa) protease complex which specifically degrades proteins conjugated to ubiquitin has previously been described, and 20S (~750 kDa) proteasome, also commonly known as macropain or the multicatalytic proteinase complex, has subsequently been shown to be the proteolytic core of the 26S complex (for reviews, see Refs. 10 and 11).

Recently, reagents which inhibit the ubiquitin-proteasome proteolytic pathway in intact cells have become available, including substrate-related peptidyl aldehydes (12-14) and a *Streptomyces* metabolite lactacystin (15). In the present study, we report that these proteasome inhibitors also considerably inhibit the degradation of autophosphorylated and polyubiquitinated PDGF β-receptors *in vivo*, suggesting the possible involvement of proteasomes in the degradation process of the ligand-activated β-receptor.

EXPERIMENTAL PROCEDURES

Chemicals: PSI (N-benzyloxycarbonyl-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal) was purchased from Peptide Institute Inc. (Osaka, Japan). Calpeptin (N-benzyloxycarbonyl-L-leucyl-L-norleucinal) was provided by T. Tsujinaka (16). Lactacystin was provided by S. Ōmura (17, 18). These drugs were dissolved in Me_2SO before use and, throughout the experiments, the final concentration of Me_2SO in cell culture media was kept 0.5 % including control cultures.

Cells: Porcine aortic endothelial (PAE) cells expressing the wild-type human PDGF β-receptor were prepared as described (19), and were cultured in Ham's F-12 medium (GIBCO) containing 10 % fetal bovine serum (GIBCO) and 200 μg/ml of the antibiotic G418 (GIBCO).

Antisera: The rabbit peptide antiserum specifically reacting with the PDGF β-receptor (PDGFR-3) was raised as described (20). The rabbit polyclonal anti-ubiquitin antiserum was purchased from Sigma. The mouse monoclonal anti-phosphotyrosine antibody (PY-20) was from Transduction Laboratories (Lexington, KY). Peroxidase-conjugated sheep anti-mouse immunoglobulins and donkey anti-rabbit immunoglobulins were from Amersham.

Ligands: Recombinant human PDGF-BB was purchased from R & D Systems (Minneapolis, MN).

Immunoblotting: Immunoblotting was performed essentially as described by Mori *et al.* (6). Confluent 25 cm^2 flasks of cells were incubated for 1 h at 37 °C with different drugs in a binding medium (Ham's F-12 medium containing 1 mg/ml of bovine serum albumin). Then the cells were cooled down on ice, and incubated in this medium with 100 ng/ml PDGF-BB for 2 h at 4 °C. After washing, the cells were further incubated at 37°C in the presence of the respective drugs for different time periods. After incubation, the cells were washed and lysed in the lysis buffer (6). The lysates were centrifuged and the supernatants were incubated with wheat germ lectin-Sepharose 6 MB (WGA-Sepharose) (Pharmacia) for 2 h at 4 °C. After washing with the lysis buffer, the beads were boiled for 3 min in the sample buffer (6) containing 2 % 2-mercaptoethanol. The samples were separated by SDS-polyacrylamide gel electrophoresis and the proteins in the gel were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham). Blots were blocked, and incubated with PDGFR-3 (1:400 dilution), PY-20 (1:1000 dilution) or the anti-ubiquitin antiserum (1:1000 dilution). The blots were washed and then incubated with the peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulins (1:5000 dilution). After washing, sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham).

RESULTS

Ligand-stimulated degradation of the PDGF β-receptor is thought to play an important role in regulation of signal transduction by the receptor. We have previously found that chloroquine, an inhibitor for lysosomal proteolysis, does not appreciably affect the rate of the receptor degradation (7). Therefore, it was of interest to clarify the mechanism of the degradation process by analyzing effects of different protease inhibitors.

First, we used a substrate-related peptidyl aldehyde, PSI, as a proteasome inhibitor (13) and examined its effect on ligand-stimulated degradation of the receptor. PAE cells expressing the wild-type PDGF β-receptor were preincubated with PSI, stimulated with PDGF-BB for 2 h at 4 °C, washed, and incubation continued at 37 °C in the presence of the drug for 0-60 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 first probed with the β-receptor specific antiserum PDGFR-3. As shown in Fig. 1, *upper panel*, the mature receptor band of 190 kDa was clearly detected in each *lane*. An upper smearing of the PDGF-BB-stimulated receptor band is due to ligand-induced receptor ubiquitination (6). When the cells were incubated at 37 °C after PDGF-BB stimulation, intensity of the smearing decreased rapidly in control cells (*lanes 2-5*), whereas the intensity decreased somewhat slowly in PSI-treated cells (*lanes 7-10*). The same blot was then stripped and reprobed with the anti-ubiquitin antiserum (Fig. 1, *middle panel*). A broad band of more than 220 kDa was visualized after PDGF-BB stimulation, which is composed of polyubiquitinated PDGF β-receptor (6). Incubation at 37 °C after PDGF-BB stimulation rapidly decreased the intensity of the ubiquitinated receptor band in control cells (*lanes 2-5*). On the other hand, in PSI-treated cells (*lanes 7-10*), the intensity became increased after 15 min of incubation and then decreased slowly with the increased incubation time. The transient increase in the intensity may be due not only to decreased efficiency in degradation of the polyubiquitinated receptor but to increased efficiency in the receptor ubiquitination at 37 °C. Finally, the blot was stripped again and reprobed with the anti-phosphotyrosine antibody PY-20 (Fig. 1, *lower panel*). A 190-kDa band with a dense smearing was observed in the sample from the PDGF-BB-stimulated cells, which corresponds to tyrosine-phosphorylated receptor. Incubation at 37 °C rapidly decreased the intensity of the band in control cells (*lanes 2-5*), whereas the intensity decreased slowly in PSI-treated cells (*lanes 7-10*). These results indicate that PSI-sensitive proteases are involved in the degradation processes of polyubiquitinated and autophosphorylated PDGF β-receptors.

In order to confirm the participation of proteasomes in the degradation process of the ligand-activated PDGF β-receptor, we used another kind of proteasome inhibitor lactacystin, which is the most specific proteasome inhibitor available to date (15). We also examined effect of another peptidyl aldehyde calpeptin, which is a specific calpain

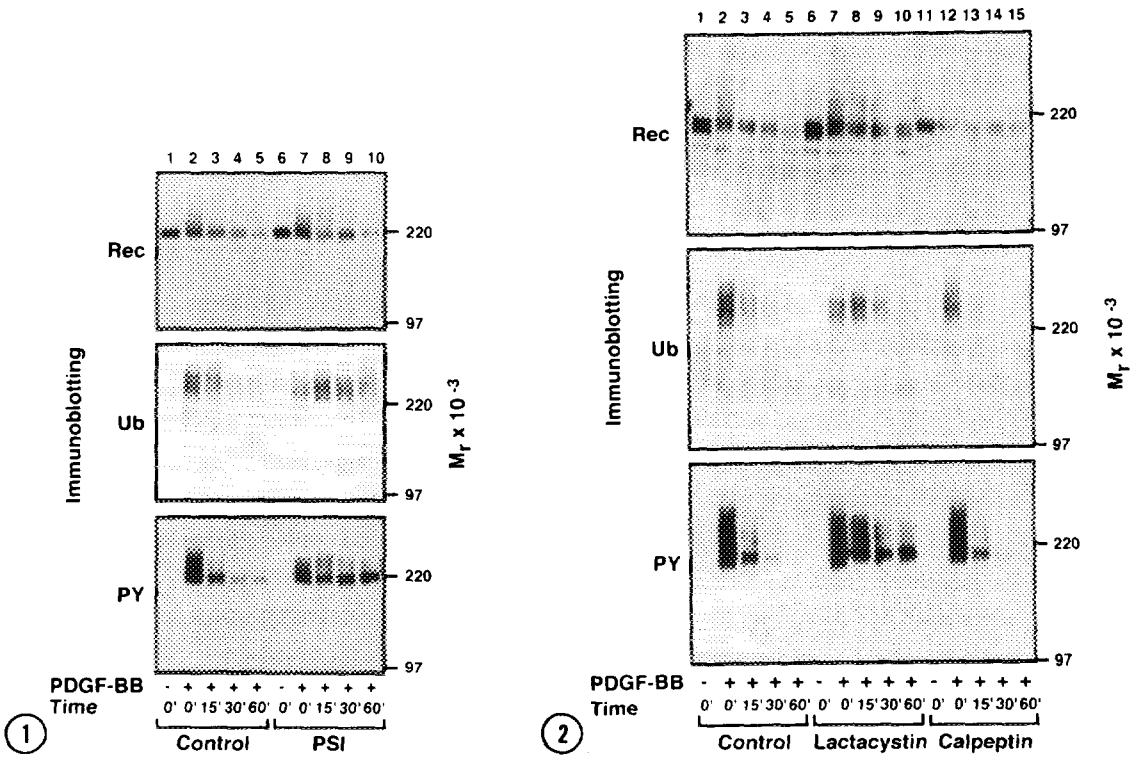

Fig. 1. Effect of PSI on ligand-stimulated degradation of the wild-type PDGF β-receptor. PAE cells expressing the wild-type human PDGF β-receptor were preincubated for 1 h at 37 °C with 0.5 % Me_2SO (*Control*) (*lanes 1-5*) or 50 μM PSI (*lanes 6-10*). Then the cells were incubated with (*lanes 2-5* and *7-10*) or without (*lanes 1* and *6*) 100 ng/ml PDGF-BB for 2 h at 4 °C, washed, and incubation continued at 37 °C in the presence of the respective drugs for the indicated time periods. 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 (*Rec*) (*upper panel*), the anti-ubiquitin antiserum (*Ub*) (*middle panel*), and PY-20 (*PY*) (*lower panel*). Sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham). The relative migration positions of molecular weight standards (myosin, 220 kDa; phosphorylase *b*, 97.4 kDa) run in parallel are indicated.

Fig. 2. Effects of lactacystin and calpeptin on ligand-stimulated degradation of the wild-type PDGF β-receptor. The wild-type receptor-expressing cells were preincubated for 1 h at 37 °C with 0.5 % Me_2SO (*Control*) (*lanes 1-5*), 100 μM lactacystin (*lanes 6-10*), or 30 μM calpeptin (*lanes 11-15*). Then the cells were incubated with (*lanes 2-5*, *7-10* and *12-15*) or without (*lanes 1*, *6* and *11*) 100 ng/ml PDGF-BB for 2 h at 4 °C, washed, and incubation continued at 37 °C in the presence of the respective drugs for the indicated time periods. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with PDGFR-3 (*Rec*) (*upper panel*), the anti-ubiquitin antiserum (*Ub*) (*middle panel*), and PY-20 (*PY*) (*lower panel*).

inhibitor (16). The wild-type receptor-expressing cells were treated with these drugs under the same conditions as those described in the previous experiment. As shown in Fig. 2, treatment of the cells with lactacystin decreased the rate of degradation of the polyubiquitinated receptor (*middle panel*, *lanes 7-10*) as well as that of the

autophosphorylated receptor (*lower panel, lanes 7-10*), as expected. On the other hand, calpeptin treatment did not affect the rate of the receptor degradation (*lanes 12-15*). These results, together with the previous results using PSI, strongly support the interpretation that proteasome-dependent proteolytic pathway is involved in the degradation process of the ligand-activated PDGF β-receptor.

DISCUSSION

Our present study demonstrates that, among different protease inhibitors examined, including PSI and lactacystin as proteasome inhibitors and calpeptin as a calpain inhibitor, only the proteasome inhibitors decrease the degradation rates of autophosphorylated and polyubiquitinated PDGF β-receptors. These data suggest that proteasome-dependent proteolysis is involved in the degradation pathway of ligand-activated β-receptor.

The proteasome inhibitors used in the present study could not completely block the receptor degradation. One possibility that the degradation process of ligand-activated PDGF β-receptor is catalyzed also by some distinct protease(s), which is resistant not only to the proteasome inhibitors but to the calpain inhibitor and chloroquine. However, a more plausible explanation is that a fraction of accumulated receptors after ligand-induced endocytosis might have become less soluble, *e.g.* through association with the detergent insoluble cell fraction, which in our procedure was removed by centrifugation from the lysate before immunoprecipitation (21).

Our previous observation that chloroquine treatment did not affect the rate of the receptor degradation (7) does not necessarily exclude the possibility that lysosomal proteolysis also contributes to ligand-stimulated degradation of the PDGF β-receptor. Because our method records the fate of the intact mature receptor molecule, that is to say the initial degradation step of the receptor, and partially cleaved receptor molecules, if any, cannot be detected due to unpredictable changes in their molecular size or immunological reactivity. It is rather conceivable that, following the proteasome-dependent degradation of the receptor, the resultant peptide fragments are delivered to and further degraded in lysosomes. The functional association of the two, apparently distinct, proteolytic systems, the ubiquitin system and the lysosomal autophagic system, has been described for the heat-induced accelerated degradation of long-lived proteins in the ts85 and the ts20 cells (which harbor a mutated thermolabile ubiquitin-activating enzyme, *E*1 (see Refs. 22 and 23)). However, the cooperative proteolysis by the proteasome and lysosome, as suggested by the present study, has not previously been reported. Our future studies will be aimed at exploring the possibility.

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