PDGF-AA and PDGF-BB Biosynthesis:
Proprotein Processing in the Golgi Complex and Lysosomal Degradation of PDGF-BB Retained Intracellularly

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Abstract. Platelet-derived growth factor is a potent mitogen for cells of mesenchymal origin. It is made up of two polypeptide chains (A and B) combined in three disulfide-linked dimeric forms (AA, AB, and BB). Here, the biosynthesis and proteolytic processing of the two homodimeric forms of PDGF (AA and BB) were studied in CHO cells stably transfected with A-chain (short splice version) or B-chain cDNA. PDGF-AA was processed to a 30-kD molecule which was secreted from the cells. In contrast, PDGF-BB formed two structurally distinct end products; a minor secreted 30-kD form and a major cell-associated 24-kD form. Immunocytochemical studies at light- and electron-microscopical levels revealed presence of PDGF in the Golgi complex, in lysosomes, and to a smaller extent in the ER. From analysis of cells treated with brefeldin A, an inhibitor of ER to Golgi transport, it was concluded that dimerization occurs in the ER, whereas the proteolytic processing of PDGF-AA and PDGF-BB precursors normally occurs in a compartment distal to the ER. Exposure of the cultures to the lysosomal inhibitor chloroquine led to an increased cellular accumulation of PDGF-BB, as determined both by metabolic labeling experiments and immunocytochemical methods, indicating that the retained form of PDGF-BB is normally degraded in lysosomes. Structural analysis of the two end products of PDGF-BB revealed that the secreted 30-kD form is a dimer of peptides processed as the B-chain of PDGF purified from human platelets, and that the retained 24-kD form is made up of subunits additionally processed in the NH$_2$-terminus. Also, the 24-kD form was shown to be composed of proteolytic fragments held together by disulfide bridges. Taken together these findings suggest that the newly synthesized PDGF A- and B-chains are dimerized in the ER and thereafter transferred to the Golgi complex for proteolytic processing. From there, PDGF-AA is carried in vesicles to the cell surface for release extracellularly by exocytosis. A smaller part of PDGF-BB (the 30-kD form) is handled in a similar way, whereas the major part (the 24-kD form) is generated by additional proteolysis in the Golgi complex, from which it is slowly carried over to lysosomes for degradation.

P LATELET-derived growth factor is a powerful mitogen for cells of mesenchymal and glial origin and has been implicated in the regulation of cell proliferation during normal as well as pathological conditions (Heldin and Westermark, 1990; Raines et al., 1990). It is composed of two polypeptide chains, A and B, that give rise to three disulfide-linked dimers, AA, AB, and BB. The A-chain and the B-chain are encoded by separate but related genes, the B-chain gene being identical to the protooncogene c-*sis*. The A-chain occurs in two variants that arise by differential splicing. The longer variant differs from the more common shorter variant by an 18–amino acid long COOH-terminal extension. The different isoforms of PDGF exert their effects on target cells by interaction with high affinity receptors of two types, the α receptor which binds either the A-chain or the B-chain, and the β receptor which binds only the B-chain (Westermark et al., 1989; Heldin and Westermark, 1990).

Both the PDGF B-chain and the short variant of the A-chain contain hydrophobic leader sequences and are synthesized as precursor molecules of 241 and 196 amino acids, respectively. Studies on cells transfected with cDNA encoding the PDGF B-chain and/or the short splice version of the A-chain have indicated that PDGF-AA and PDGF-AB are trimmed proteolytically after dimerization and secreted as 30-kD products, whereas PDGF-BB is processed into a minor secreted product of 30 kD and a major cell-associated product of 24 kD (Robbins et al., 1983; Beckmann et al., 1988; Bywater et al., 1988; Östman et al., 1988). Electron microscopic and cytochemical studies of transfected CHO cells have revealed that only small amounts of PDGF-AA are present intracellularly, mainly in the ER and in secretory vacuoles. On the other hand, PDGF-BB occurs in diluted form in the ER, is highly concentrated in the Golgi complex, and is at least in part transferred to the lysosomes (Thyberg

et al., 1990). Amino acid sequencing of the secreted forms and of peptides derived from human platelet PDGF-AB have shown that the B-chain of the 30-kD PDGF forms consists of amino acids 82–190 of the precursor and thus represents a form that is both NH_2- and COOH-terminally trimmed (Johnsson et al., 1984). In contrast, the A-chain appears to be cleaved only NH_2 terminally.

Analysis of chimeras between the short splice version of the PDGF A-chain and the B-chain has recently localized the structural determinant for cellular retention of PDGF-BB to amino acids 219–229 within the COOH-terminal propart of the PDGF B-chain precursor (LaRochelle et al., 1991; Östman et al., 1991). Interestingly, this sequence is homologous to the unique COOH-terminal sequence of the long splice version of the PDGF A-chain, as well as to a sequence within the longer variant of VEGF/VPF, a recently identified endothelial cell growth factor (Keck et al., 1989; Leung et al., 1989). Analysis of the processing of the long splice version of the A-chain, transiently expressed in COS cells, also revealed a processing pattern very similar to that of the PDGF B-chain (Östman et al., 1991). However, the detailed functional and structural properties, as well as the intracellular fate of the retained forms, remain unknown.

Here, the processing of PDGF-AA and PDGF-BB were explored in further detail. Processing intermediates were identified by pulse–chase analysis and their subcellular localization determined by immunofluorescence and immunoelectron microscopy. Cells treated with brefeldin A, a fungal metabolite that has been found to inhibit ER to Golgi transport, carbohydrate trimming and proteolytic cleavage of secretory proteins (Klausner et al., 1992), was used to identify the subcellular localization of the proteolytic processing of the precursor forms of PDGF-AA and PDGF-BB. The role of the lysosomes in the degradation of PDGF-BB retained intracellularly was determined by studying cultures treated with chloroquine, a weak base that raises intralysosomal pH and inhibits lysosomal degradation of organic molecules (Mellman et al., 1986). Finally, the two different PDGF-BB end products were structurally characterized and compared.

Materials and Methods

Transfected Cell Lines

CHO (dhfr-) cells (DxB11) were cotransfected with the plasmid pAd (Chiron Corporation, Emeryville, CA), which contains the mouse dihydrofolate reductase gene under control of the adenovirus major late promoter, and either the pSV7d-PDGF-A102 or the pSV7d-PDGF-B1 plasmids (Östman et al., 1988), which encode the shorter variant of the human PDGF A-chain and the human PDGF B-chain, respectively. Transfection, selection, and isolation of the cell lines CHO–PDGF-A and CHO–PDGF-B (c-sis45), which expressed high levels of PDGF-AA and PDGF-BB, respectively, were made as described (Östman et al., 1988).

Construction, Expression, and Analysis of PDGF Mutants PDGFB-R113A and PDGFB-R108, 109, 113A

Site-directed mutagenesis was performed by the method of Kunkel et al. (1987). BglII/EcoRI fragments in which Arg-113 was mutated to an alanine residue (PDGFB-R113A) or in which Arg-108, -109, and -113 were mutated to alanine residues (PDGFB-R108, 109, 113A) were inserted into the vector pSV7d/PDGF-B1 (Östman et al., 1989), from which the corresponding wild-type sequence had been excised. Transfections of COS cells and analy-

sis of transiently expressed proteins were performed as described (Östman et al., 1991).

Drugs and Immunological Reagents

Brefeldin A (Epicentre Technologies, Madison, WI) was used at a final concentration of 10 μM (diluted from a 4.0 mM stock solution in ethanol) and chloroquine at 100 μM (diluted from a 5.0 mM stock solution in medium F-12). The antisera against human platelet PDGF and recombinant PDGF-AA and PDGF-BB have been described previously (Heldin et al., 1981; Thyberg et al., 1990). A mouse mAb against a synthetic fragment of the PDGF B-chain (Shiraishi et al., 1989) was kindly provided by Mochida Pharmaceutical Company (Tokyo, Japan) and rabbit antibodies against mannosidase II (Moremen et al., 1991) by Dr. K. W. Moremen (University of Georgia, Athens, GA) and Dr. M. G. Farquhar (University of California San Diego, La Jolla, CA). Rhodamine- and fluorescein-conjugated swine immunoglobulins to rabbit immunoglobulins were obtained from Dako Corp. (Glostrup, Denmark), rhodamine-conjugated goat immunoglobulins to mouse immunoglobulins from Sigma Chemical Co. (St. Louis, MO), and peroxidase-conjugated, affinity-purified F(ab')₂ fragments of goat anti-rabbit IgG from Immunotech (Marseille, France).

Immunoprecipitation of Metabolically Labeled CHO–PDGF-A and CHO–PDGF-B Cells

Cells grown to confluence in 25-cm² plastic flasks were labeled with 0.3 mCi [³⁵S]cysteine in 1.5 ml cysteine-free medium supplemented with 10% dialyzed FCS, washed with cysteine-free medium, and chased in medium containing 250 μg/ml unlabeled cysteine. Chase medium and cell lysates were subjected to immunoprecipitation with anti–PDGF-AA or anti–PDGF-BB and the precipitates were analyzed by SDS-PAGE and fluorography as described (Östman et al., 1988).

Cyanogen Bromide Cleavage of [³⁵S]Cysteine-labeled and Immunoprecipitated PDGF-BB

CHO–PDGF-B cells were labeled with [³⁵S]cysteine for 3 h and chased in nonradioactive medium for 2 h as described above; cell lysates and conditioned media were then collected and immunoprecipitated using a PDGF antiserum (Heldin et al., 1981). The precipitated material was incubated with 100 μl 50% protein A-Sepharose slurry in PBS, pH 7.3, washed, and collected by centrifugation. Formic acid was added to the samples, either alone (final concentration 70%, total vol 300 μl) or together with CNBr (final concentration 10 mg/ml). The protein A-Sepharose beads were pelleted by centrifugation and the supernatants were incubated for 24 h at 20°C. The samples were dried in a SpeedVac concentrator, dissolved in 200 μl SDS-sample buffer (Blobel and Dobberstein, 1975) without reducing agent, and heated to 95°C for 3 min. A 100-μl aliquot of each sample was reduced by incubation in 10 mM DTT for 90 s at 95°C and alkylated by addition of iodoacetamide to a final concentration of 50 mM and continued incubation at 20°C for 15 min. The samples were finally analyzed by SDS-PAGE and fluorography (Östman et al., 1988).

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy, cells grown on glass coverslips were fixed in 2% formaldehyde in PBS, pH 7.3, for 30 min at 20°C, quenched in 50 mM NH₄Cl in PBS (15 min), permeabilized with 0.05% Triton X-100 in PBS (30 min), exposed to primary and secondary antibodies (2–3 h each) in PBS/0.1% BSA, and finally mounted in 90% glycerol containing 0.1% p-phenylenediamine. The specimens were examined in a Nikon Labophot microscope (Nikon Inc., Melville, NY) with epifluorescence optics (Tri-X pan film; Eastman Kodak Co., Rochester, NY). In the double staining experiments the cells were fixed in acetone/methanol (1/1 by vol) for 5 min at 20°C and thereafter rinsed twice in PBS. Otherwise, the procedure was the same as described above.

Immunoelectron Microscopy

Immunoperoxidase staining was performed as described (Brown and Farquhar, 1989; Thyberg et al., 1990). Shortly, the cells were fixed in phosphate-buffered 2% formaldehyde/0.05% glutaraldehyde (60 min), rinsed with PBS/0.1% BSA, and permeabilized with 0.05% saponin in PBS/0.1% BSA (5 min). They were exposed to primary antibodies and peroxidase-conjugated goat anti–rabbit F(ab')₂ in PBS/0.1% BSA/0.05% saponin

(90–120 min each). After rinsing with PBS (with and without saponin), the cells were fixed in 3% buffered glutaraldehyde (30 min) and rinsed again with PBS. Peroxidase activity was visualized by incubation with 0.5 mg/ml DAB tetra-HCl (Sigma Chemical Co.) plus 0.01% H_2O_2 in PBS (10–20 min). The cells were then scraped off the petri dishes in 0.1 M sodium cacodylate-HCl buffer, pH 7.3, and transferred to plastic tubes, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate-HCl buffer, pH 7.3, containing 0.5% potassium ferrocyanate (60 min at 4°C), dehydrated in ethanol, stained with 2% uranyl acetate in ethanol, and embedded in low-viscosity epoxy resin. Thin sections were cut on an LKB Ultratome IV and examined in an electron microscope (model 100CX; Jeol U. S. A. Inc., Peabody, MA) at 60 kV (Kodak 4489 film; Eastman Kodak Co.).

Results

Processing and Subcellular Localization of PDGF-AA and PDGF-BB in Transfected Cells

To study the processing and assembly of PDGF-AA and PDGF-BB, metabolic labeling and immunoprecipitation experiments were performed on two transfected CHO cell lines, CHO–PDGF-A and CHO–PDGF-B. These cell lines were obtained by cotransfecting an expression vector containing the coding region of the shorter variant of the human PDGF A-chain and the PDGF B-chain, respectively, with an expression vector containing the mouse dihydrofolate reductase (dhfr) cDNA into CHO (dhfr-) cells. After a 2 h pulse with [³⁵S]cysteine, the cells were chased for various periods of time in medium containing an excess of unlabeled cysteine. The cell lysates and the corresponding media were then subjected to immunoprecipitation with rabbit PDGF antisera and the immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Unreduced gels of material precipitated from the medium showed that already at the end of the radioactive pulse, the major part of PDGF-AA was secreted as species of 40, 35, and 30 kD (Fig. 1, B and E); during the subsequent chase, the larger forms in the medium were converted into the 30-kD form. All these three forms, as well as a 23-kD form, were also present in the cell lysate (Fig. 1 B). The latter species was seen only at the earliest time point studied and most likely represents an undimerized precursor form, since reduced gels of the cell lysates of the earliest time point revealed a major band of 23 kD and a doublet of 16–17 kD (Fig. 1 A). On the basis of these observations, it is concluded that the 40-kD form corresponds to a dimer composed of uncleaved 23-kD PDGF A-chain precursors, whereas the 30-kD form is a dimer of processed 16–17-kD chains. The 35-kD form most likely represents an intermediate where only one of the subunits is processed.

In the case of PDGF-BB, unreduced gels showed an initial 56-kD dimeric form in the cell lysate, which via an intermediate of 40 kD, was converted into a final product of 24 kD (Fig. 1 I). When the gels were run under reducing conditions, monomeric forms of 28, 22, and 12 kD were found (Fig. 1 H). Under the exposure conditions used in the experiment illustrated in Fig. 1, no secreted forms of PDGF-BB were observed. However, films exposed for longer times revealed in the medium at the earliest time points small amounts of dimeric species with sizes between 56 and 40 kD in addition to a 30-kD form; as the chase continued, the higher molecular weight species disappeared and the 30-kD form accumulated (data not shown). Comparisons of autoradiograms exposed an equally long time revealed that the

24-kD form of PDGF-BB was at least 10-fold as abundant as the secreted 30-kD form. Thus, the product of the transfected PDGF B-chain cDNA is also dimerized before proteolysis and forms a 56-kD precursor, which via an intermediate of 40 kD, is processed into two final forms, a major cell-associated form of 24 kD and a minor secreted form of 30 kD.

The intracellular localization of PDGF-AA and PDGF-BB in the transfected cells was studied by indirect immunofluorescence microscopy and immunoperoxidase staining in combination with EM. CHO–PDGF-A cells showed a weak juxtanuclear reactivity with anti–PDGF-AA in the fluorescence microscope (Fig. 2 A). With the immunoperoxidase method, only little reactivity for PDGF-AA could be detected in these cells (see Thyberg et al., 1990). In CHO–PDGF-B cells a juxtanuclear staining of somewhat variable extension was demonstrated with anti–PDGF-BB by fluorescence microscopy (Fig. 2 C). At the electron microscopic level, reaction product was most prominent in stacked Golgi cisternae (Fig. 3 A). However, there was also a positive staining of lysosome-like vacuoles and, more occasionally, a weak staining of the ER (see Thyberg et al., 1990). With antibodies against mannosidase II, an established marker of the Golgi complex (Novikoff et al., 1983; Moremen and Touster, 1988), the staining was more clearly restricted to the stacked Golgi cisternae (Fig. 3 B). Double immunofluorescence microscopy with the monoclonal PDGF B-chain antibody and anti-mannosidase II confirmed that PDGF-BB was present not only within but also outside the Golgi complex, namely in numerous vesicles presumably representing endosomes and/or lysosomes (Fig. 2, E and F).

Effects of Brefeldin A on Processing, Turnover, and Localization of PDGF-AA and PDGF-BB

To identify the subcellular compartment where the proteolytic processing of PDGF-AA and PDGF-BB takes place, the transfected cells were treated with brefeldin A and subjected to pulse–chase analysis as described above. In CHO–PDGF-A cells, brefeldin A was found to strongly inhibit the proteolytic processing of PDGF-AA and totally block its secretion into the medium (Fig. 1, C and F). In CHO–PDGF-B cells, brefeldin A slowed down the conversion of the precursor into smaller forms (Fig. 1 J). The 40-kD form, which in untreated cells was the predominant form after 2 h of chase (Fig. 1 I), was detected in the brefeldin A-treated cells only after 8 h of chase (Fig. 1 J). In the brefeldin A-treated cells, the major component was initially 56 kD, like the precursor of untreated cells. However, with time this species increased in size, most likely because of posttranslational modifications.

Thus, brefeldin A did not affect the dimerization of the newly synthesized PDGF A- and B-chains but markedly interfered with the proteolytic processing of PDGF-AA as well as PDGF-BB. We therefore conclude that dimerization occurs already in the ER, whereas the proteolytic processing takes place in compartments distal to the block induced by brefeldin A. Since in untreated cultures precursor and mature forms of PDGF-AA were found both in the cells and in the medium, it seems likely that the processing in this case may take place either in the Golgi complex or later on along the secretory pathway, i.e., in secretory vacuoles or extracellularly. With regard to PDGF-BB, the proteolytic processing seems more restricted to the Golgi complex.
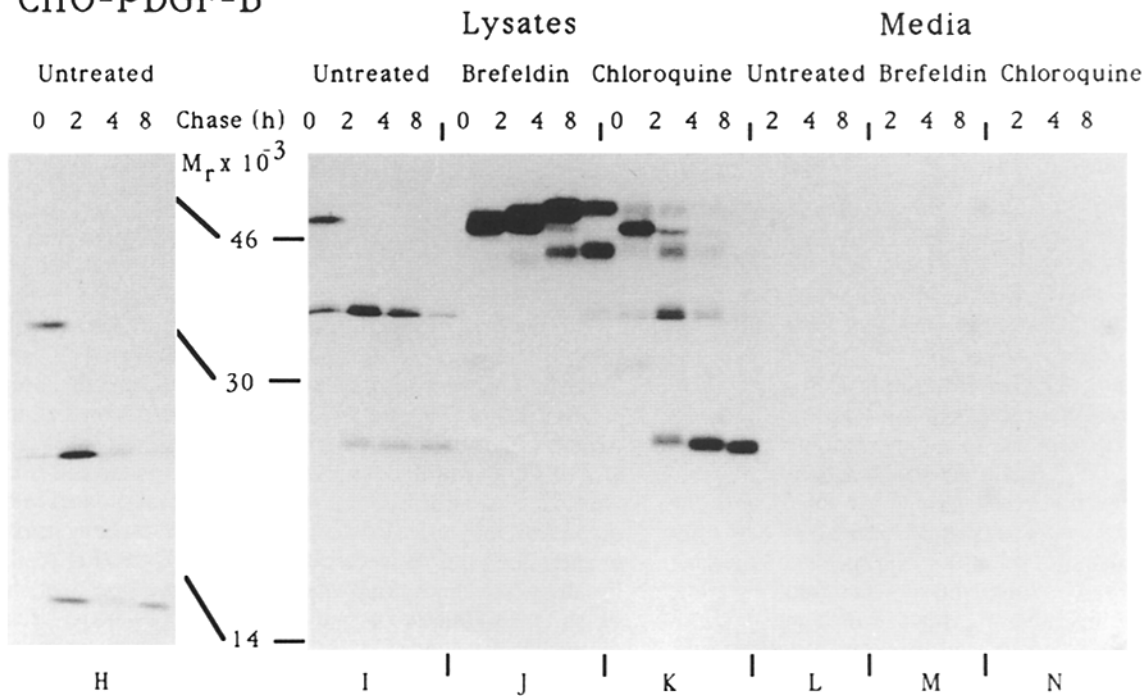
CHO-PDGF-A
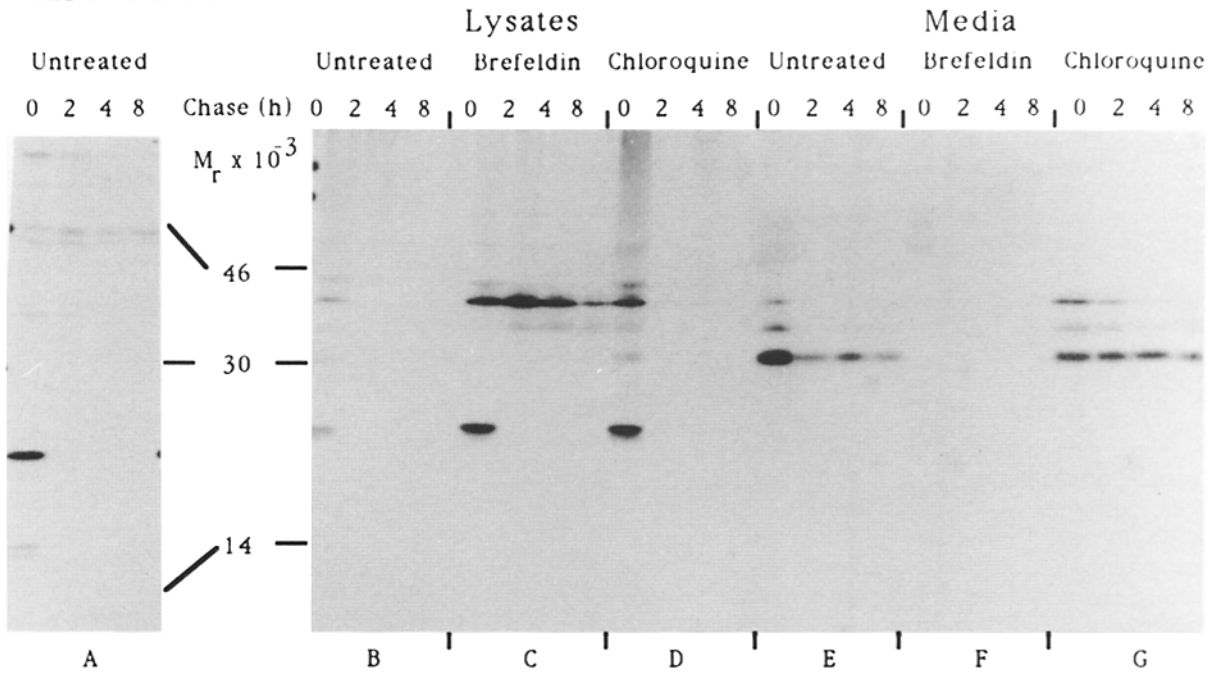
CHO-PDGF-B

Figure 1. Pulse–chase analysis of the processing of PDGF-AA and PDGF-BB in untreated metabolically labeled cells and cells treated with 10 μM brefeldin A or 100 μM chloroquine. CHO–PDGF-A and CHO–PDGF-B cells were labeled for 2 h with [^{35}S]cysteine and then grown for the indicated time periods in medium containing an excess of unlabeled cysteine. Cell lysates and conditioned media were subjected to immunoprecipitation using anti–PDGF-AA or anti–PDGF-BB and the precipitates were analyzed by SDS-PAGE under nonreducing (*B–G* and *I–N*) conditions followed by fluorography. *A* and *H* show the result of SDS-PAGE under reducing conditions of the samples shown in *B* and *I*, respectively. Positions of molecular weight markers are indicated between *A* and *B* and *H* and *I*.
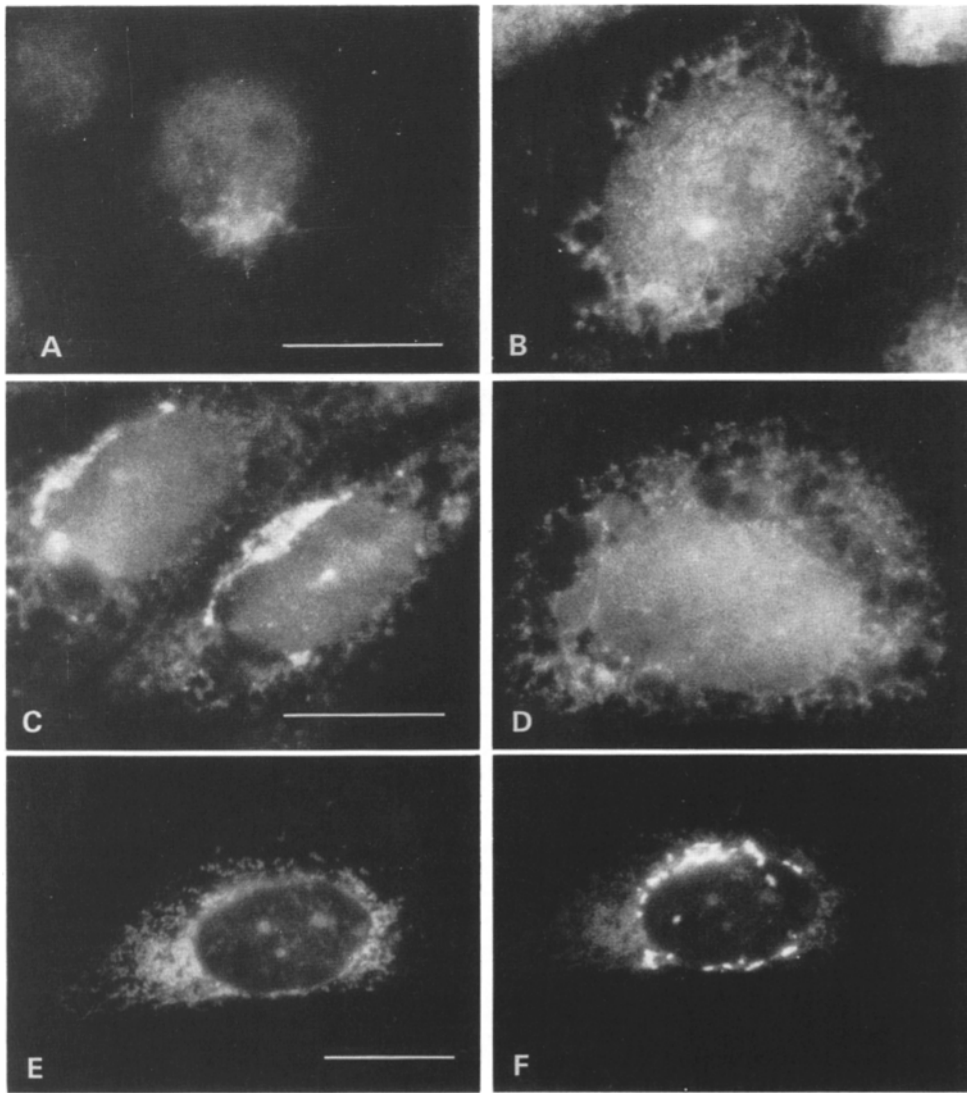
Figure 2. Immunofluorescence microscopy of CHO–PDGF-A cells (*A* and *B*) and CHO–PDGF-B cells (*C–F*) kept in normal medium (*A, C, E,* and *F*) or treated with 10 μM brefeldin A for 30 (*B*) or 120 (*D*) min. Single staining with rabbit anti–PDGF-AA (*A* and *B*) or anti–PDGF-BB (*C* and *D*), or double staining with mouse anti–PDGF B-chain (*E*) and rabbit anti–mannosidase II (*F*). Bars, 20 μm.

To localize the PDGF forms in cells treated with brefeldin A, CHO–PDGF-A and CHO–PDGF-B cells were stained for indirect immunofluorescence microscopy. After treatment with 10 μM brefeldin A for 30 or 120 min, the staining of CHO–PDGF-A cells was spread throughout large parts of the cells, often showing a reticular pattern (Fig. 2 *B*). If the cells were exposed to the drug for 120 min, rinsed, and then incubated in drug-free medium for 60 min, the staining pattern was normalized. Treatment of CHO–PDGF-B cells with 10 μM brefeldin A turned the Golgi-like staining into a dispersed ER-like pattern. The changes were evident after 30 min and fully developed after 120 min of drug exposure (Fig. 2 *D*). Upon removal of brefeldin A, a juxtanuclear distribution of the reaction product was reestablished.

Brefeldin A-treated cells were also studied by EM. In CHO–PDGF-A cells, treatment with 10 μM brefeldin A for 120 min gave rise to a weak staining in the ER in part of the cells (not shown). Treatment of CHO–PDGF-B cells with 10 μM brefeldin A led to distinct changes, including a prominent increase in overall reactivity with anti–PDGF-BB and a redistribution of reactive material from the Golgi complex

to the ER. After 30 min of exposure to the drug, the Golgi stacks were to a large extent disintegrated into vesicular and tubular elements and reaction product for PDGF-BB occurred in some of these elements as well as in ER cisternae (Fig. 4 *A*). After 120 min, no recognizable Golgi stacks were noted and the vesiculotubular structures remaining in the former Golgi area were negative for PDGF-BB (Fig. 4 *B*). On the other hand, large amounts of reaction product was found in ER cisternae throughout the cytoplasm, including the nuclear envelope (Fig. 4 *C*).

Chloroquine Causes Accumulation of 24-kD PDGF-BB in Lysosomes

Our previous studies have indicated that PGDF-BB is retained intracellularly and is at least in part transferred to lysosomes (Thyberg et al., 1990). To investigate if these organelles represent the normal site of degradation of the protein, the cultures were treated with the weak base chloroquine, a well established inhibitor of lysosomal proteolysis (Mellman et al., 1986). In pulse–chase experiments with
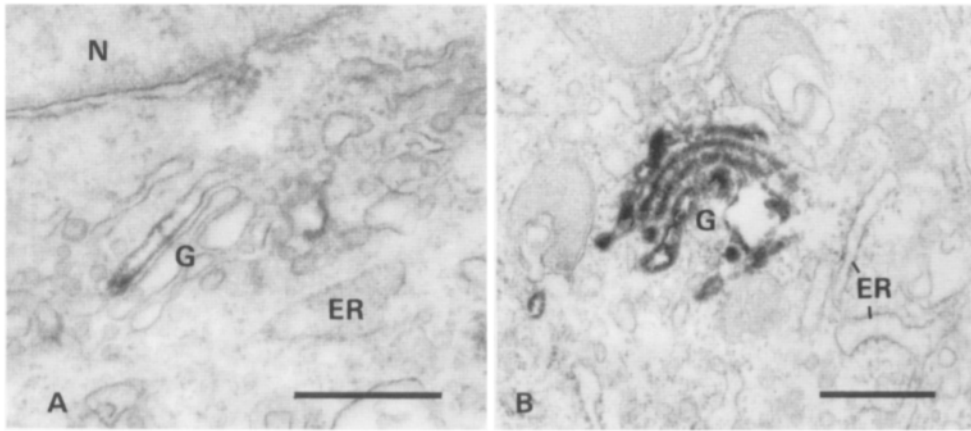
Figure 3. Immunoperoxidase localization of PDGF-BB (*A*) and mannosidase II (*B*) in CHO–PDGF-B cells kept in normal medium. Reaction product is observed in stacked Golgi cisternae (*G*). *ER*, endoplasmic reticulum; *N*, nucleus. Bars, 0.5 μm.
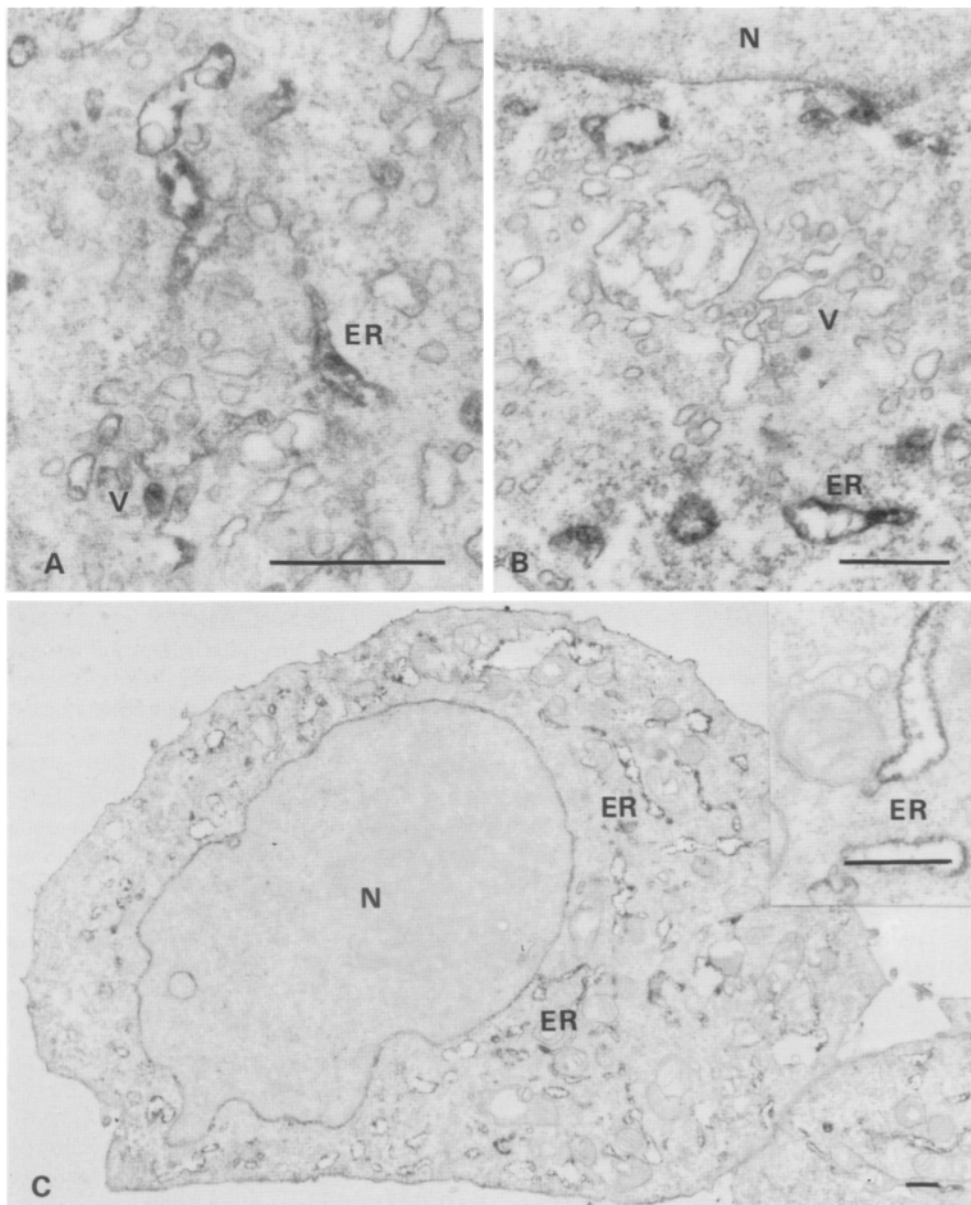

Figure 4. Immunoperoxidase localization of PDGF-BB in CHO–PDGF-B cells exposed to 10 μM brefeldin A for 30 (*A*) or 120 (*B* and *C*) min. After 30 min, staining for PDGF-BB appears in the *ER* and in a few tubulovesicular structures (*V*) in the former Golgi area (*A*). After 120 min, most of the latter structures are negative (*B*), whereas a positive reaction is found throughout large parts of the ER (*C*). *N*, nucleus. Bars, 0.5 μm.
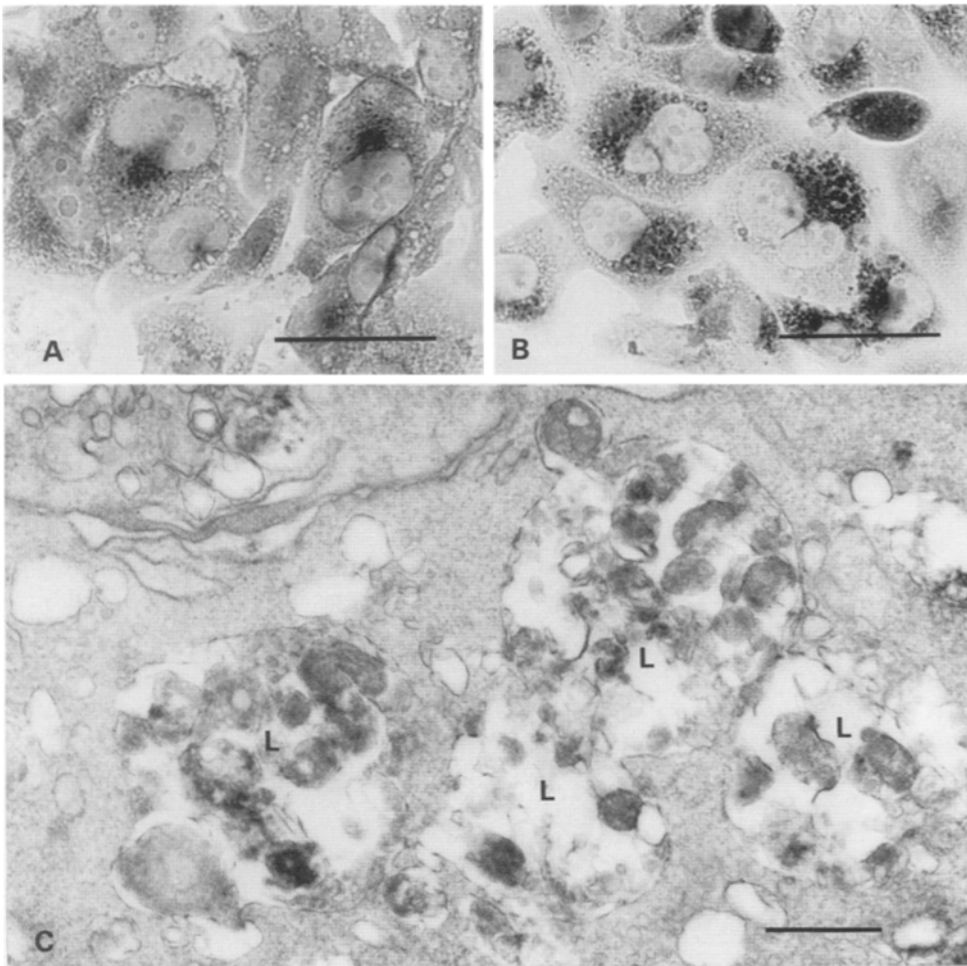
Figure 5. Effect of chloroquine on the distribution of PDGF-BB in CHO–PDGF-B cells as studied by light microscopy (*A* and *B*) and EM (*C*). Control cultures (*A*) and cultures exposed to 100 μM chloroquine for 15 h (*B* and *C*) were fixed and stained for PDGF-BB using the immunoperoxidase technique. At the electron microscopic level (*C*), reaction product is found in swollen lysosomes (*L*) with inclusions of variable appearance. Bars: (*A* and *B*) 20 μm; (*C*) 0.5 μm.

metabolically labeled CHO–PDGF-B cells, chloroquine slowed down the disappearance of labeled forms as compared with untreated cells (Fig. 1, *I* and *K*). However, the pattern of processing was not changed, suggesting that the proteolytic steps leading to formation of the 40- and 24-kD forms occurred in compartments insensitive to chloroquine. Furthermore, the amount of PDGF-BB secreted into the medium was unaffected by chloroquine, as determined by
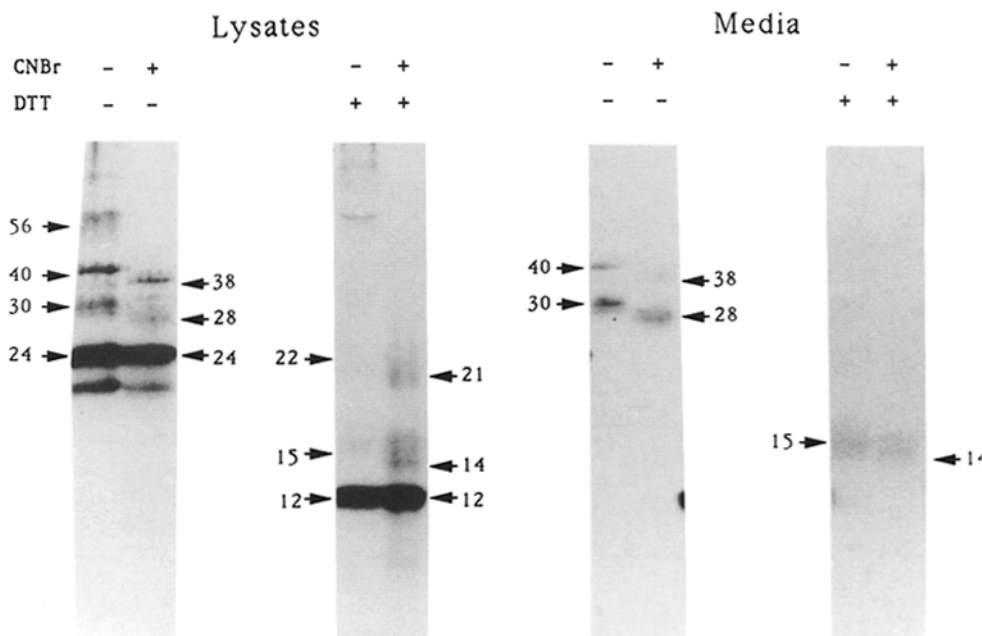

Figure 6. CNBr cleavage of PDGF-BB. PDGF-BB immunoprecipitated from metabolically labeled CHO–PDGF-B cells, or the corresponding conditioned medium, was treated with 70% formic acid alone or 70% formic acid containing 10 mg/ml CNBr. The samples were analyzed by SDS-PAGE under nonreducing or reducing conditions, followed by fluorography. Arrowheads indicate the different forms of PDGF-BB and their apparent sizes expressed in kilodaltons.

films exposed for longer times (data not shown). In CHO–PDGF-A cells, no distinct effects on the assembly, processing, and secretion of PDGF-AA were detected after treatment with chloroquine (Fig. 1, *D* and *G*).

Immunocytochemical analysis of CHO–PDGF-B cells exposed to chloroquine for 4–24 h revealed a gradual accumulation of vacuoles filled with PDGF-BB (Fig. 5, *A* and *B*). In the electron microscope, these vacuoles were identified as lysosomes filled with inclusions of incompletely degraded material (Fig. 5 *C*). In CHO–PDGF-A cells, no intracellular accumulation of PDGF-AA could be detected after chloroquine treatment.

Structural Differences Between the 24- and 30-kD Forms of PDGF-BB

The finding of two structurally different dimeric end products of PDGF-BB prompted us to compare them structurally. Using a procedure developed for the purification of PDGF-AA secreted from a human osteosarcoma cell line (Heldin et al., 1986), the secreted form was purified from serum-free conditioned media. By NH₂-terminal amino acid sequencing the secreted PDGF-BB was found to display the same NH₂-terminus as the B-chain of platelet PDGF-AB, i.e., starting at position 82 of the precursor molecule (data not shown). When analyzed by SDS-PAGE, the secreted form also appeared very similar to recombinant PDGF-BB produced in yeast (Östman et al., 1989), which is composed of subunits containing amino acids 82–190 of the PDGF B-chain precursor (data not shown). Hence, we conclude that the 30-kD form is a dimer composed of two subunits processed as the B-chain in human platelet PDGF-AB. This form of secreted PDGF-BB has also been identified as one of the PDGF isoforms present in the conditioned media of CHO cells stably expressing both the PDGF B-chain and the short splice version of the A-chain (Östman et al., 1988).

To elucidate the structure of the 24-kD form relative to the 30-kD form, the various forms of PDGF-BB were subjected to CNBr cleavage, the rationale being that only forms retaining any of the methionine residues in the PDGF B-chain precursor (amino acids 64 and 93) would shift in size after cleavage. CHO–PDGF-B cells were metabolically labeled with [³⁵S]cysteine; the medium and cell lysate were then subjected to immunoprecipitations. The samples were split in two parts and treated either with CNBr in 70% formic acid or 70% formic acid only, and then analyzed by SDS-PAGE and fluorography. The 56-, 40-, 30-, and 24-kD forms could be identified in the immunoprecipitates treated with formic acid only (indicated by *arrowheads* in Fig. 6); all these species except the 24-kD form shifted in mobility after exposure to CNBr (Fig. 6). This suggests that the 24-kD form, in contrast to the 30-kD form, lacks the methionine at position 93. Analysis of the CNBr-treated samples under reducing conditions gave results consistent with this interpretation; only the 12-kD from was resistant to CNBr (Fig. 6). In agreement with this finding, it was not possible to detect the 24-kD form in immunoprecipitates of cells labeled with [³⁵S]methionine (data not shown).

Amino acid sequencing of the 30-kD form of PDGF-BB has shown that proteolytic processing after the arginine residue at position 113 occurs with high frequency (Östman et al., 1988, 1989; Hart et al., 1990). To investigate whether proteolysis at that position is involved in the generation of the 24-kD form of PDGF-BB, two variants of the PDGF B-chain cDNA were made by site-directed mutagenesis. In PDGFB-R113A codon 113 was changed to encode alanine
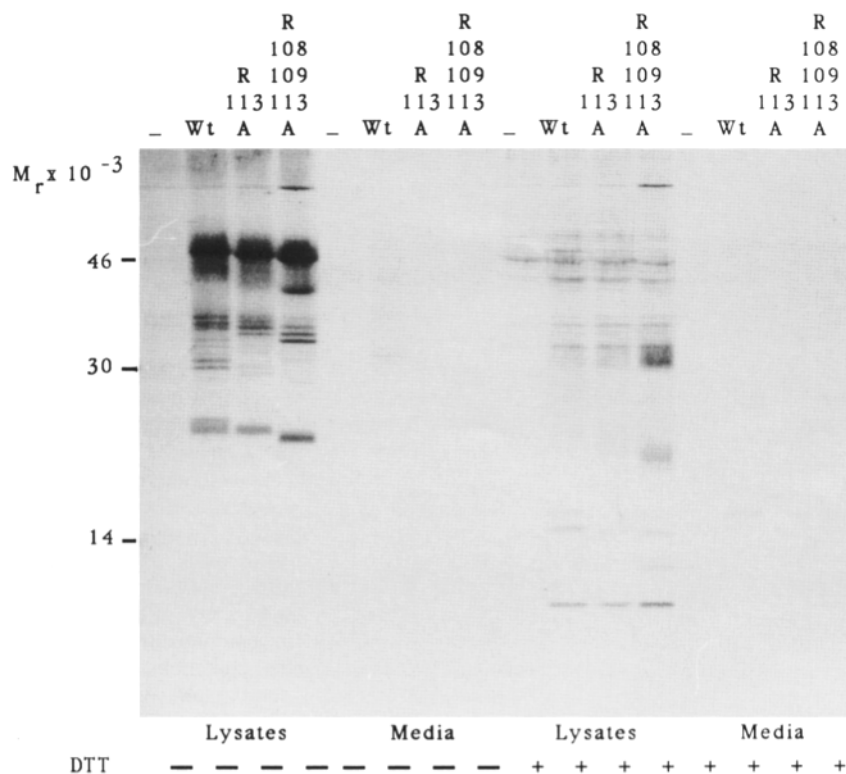

Figure 7. Expression in COS cells and immunoprecipitations of wild-type PDGF B-chain (*Wt*) and the mutants PDGFB-R113A and PDGFB-R108,109,113A. Transfected and mock-transfected (−) COS cells were metabolically labeled with [³⁵S]cysteine for 4 h and chased in medium containing excess unlabeled cysteine for 4 h. Lysates and conditioned media were immunoprecipitated with anti–PDGF-BB and analyzed by SDS-PAGE under reducing and nonreducing conditions.

instead of arginine and in PDGFB-R108,109,113A, the three arginine residues 108, 109 and 113 were changed to alanine residues. These cDNAs were cloned into the vector pSV7d and the encoded proteins were transiently expressed in COS cells and analyzed by immunoprecipitations and SDS-PAGE.

As shown in Fig. 7, the amount and structure of the secreted forms was similar for wild-type and mutated variants of PDGF-BB. In all cases a 24-kD cell-associated form appeared under nonreducing conditions. A small difference in migration between the wild-type and mutated cell-associated 24-kD forms could be observed, most likely because of the changes of the positively charged arginine residues to alanine residues, indicating that the mutated region is part of the 24-kD form. In contrast, no difference in migration was observed in the reduced 12-kD forms derived from the wild-type and mutated variants, indicating that they do not contain the mutated region.

Our data thus suggest that the polypeptide chains of the 24-kD component is additionally processed in the NH$_2$ terminus and composed of proteolytic fragments held together by disulfide bridges (Fig. 8 and see below).

Discussion

The assembly, biosynthesis, and processing of PDGF-AA and PDGF-BB, as determined in the present study, are schematically illustrated in Fig. 8. The A-chain (short splice version) and the B-chain precursors of PDGF are both synthesized and dimerized in the ER to form precursor molecules of 40 and 56 kD, respectively. PDGF-AA is then transported to the Golgi complex and follows the default pathway for secretion. Proteolytic processing of PDGF-AA into the final 30-kD product takes place in the Golgi complex or further on along the secretory route. Since all forms of dimeric

PDGF-AA were also seen in the conditioned media we conclude that proteolytic processing of the precursor form of the dimer is not a requirement for secretion. PDGF-BB is likewise carried from the ER to the Golgi complex. Here, a major part is processed to a 24-kD component that accumulates in the Golgi cisternae before it is transferred to the lysosomes and degraded.

The identification of the compartment in which the 24-kD form of PDGF-BB is degraded as lysosomes relies on a number of observations. PDGF-BB can normally be observed in the lysosomes of cells transfected with B-chain cDNA (Thyberg et al., 1990). Moreover, the weak base chloroquine, an inhibitor of lysosomal proteolysis (Mellman et al., 1986), slowed down the disappearance of metabolically labeled PDGF-BB from the cells, without interfering with the generation of the 24-kD form of the molecule or the secretion of the 30-kD form (Fig. 1 K). At the same time, material reactive with anti-PDGF-BB accumulated in vacuoles with morphological characteristics of lysosomes (Fig. 5). In contrast, many proteins which fail to fold correctly or to assemble into the proper oligomeric complexes have been found to be retained intracellularly and degraded in a pre-Golgi compartment insensitive to chloroquine and other lysosomal inhibitors (Hurtley and Helenius, 1989; Klausner and Sitia, 1990). Since no clear effects of chloroquine were seen on the processing of PDGF-AA in cells transfected with A-chain cDNA (Fig. 1, D and G), the effect of chloroquine can not be attributed to a general decrease in the rate of transfer of proteins between subcellular compartments. Furthermore, since these cells do not express PDGF receptors (Claesson-Welsh et al., 1988) the generation and lysosomal degradation of the 24-kD form is not a consequence of receptor-mediated uptake.
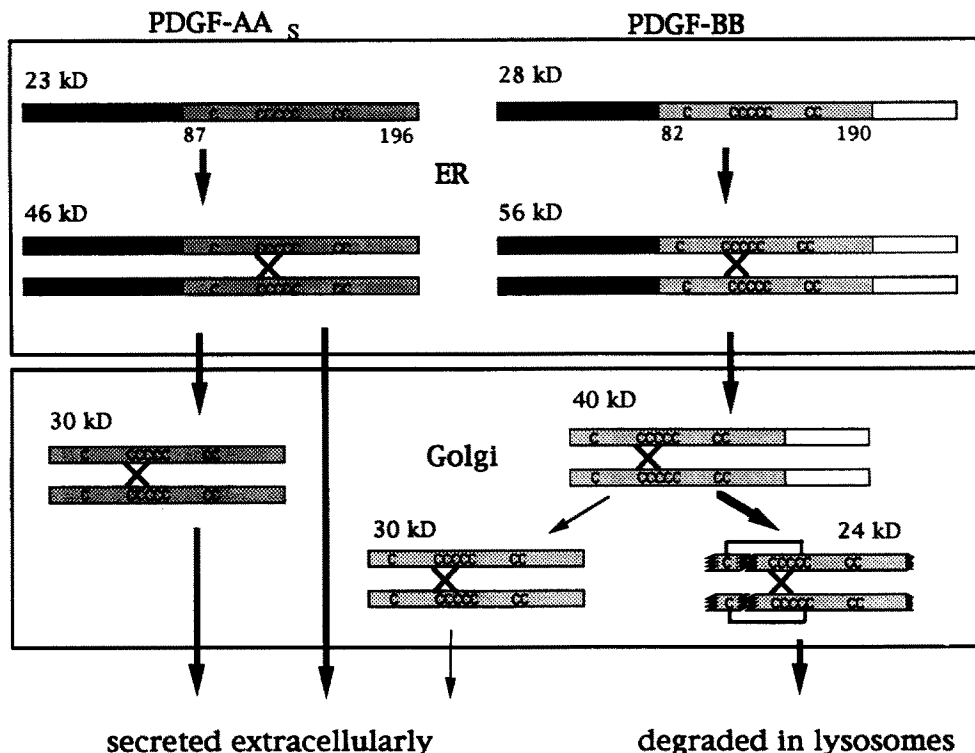

Figure 8. Schematic illustration of the assembly, intracellular transport, and processing of PDGF-AA and PDGF-BB in transfected CHO cells. See the text for discussion. The interchain disulfide bridges are depicted as determined by Andersson et al. (1992). Other disulfide bonds in PDGF have not been determined. Part of the processing of the 40-kD form of PDGF-BB to the 30-kD form may also occur extracellularly in certain cell types (La Rochelle et al., 1991; Raines and Ross, 1992).

Due to the difficulties to obtain sufficient amounts of pure 24-kD form for protein sequencing, the detailed structural characteristics of the 24-kD form of PDGF-BB remain unknown. However, our data show that the 24-kD form does not contain the methionine residue at position 93 and is thus processed NH₂-terminally in a different way compared with the 30- and 40-kD form (Fig. 6). Also, the studies on PDGF B-chain mutants with arginine residues changed to alanine residues indicate that the conversion from the 30 to the 24-kD form is not mediated through proteolysis at arginine residues 108, 109, or 113 (Fig. 7). Finally, the shift in migration in SDS-PAGE observed for the mutated nonreduced 24-kD forms, but not for the reduced 12-kD forms, suggest that the three arginine residues at position 108, 109, and 113 are present in the nonreduced 24-kD components, but not in the reduced 12-kD components (Fig. 7). One possible interpretation of these observations is that the conversion from the 30- to the 24-kD form involves proteolysis in the NH₂-terminus, as indicated by the absence of Met-93 in the 24-kD form, and in addition somewhere between Arg-113 and the second cysteine (Cys-124). The most NH₂-terminal cysteine residue (Cys-97) would then be disulfide linked to another of the cysteine residues further down in the sequence and therefore be present in the nonreduced 24-kD form, but not in the reduced 12-kD molecule (Fig. 8). It is not known if the COOH termini of the 24- and the 30-kD form are similar. However, this seems likely since the 12-kD reduced form is recognized by a peptide antiserum raised against a peptide encompassing amino acids 154–170 of the B-chain precursor (Östman et al., 1988), but not by a peptide antiserum raised against the COOH-terminal propart of the precursor (Robbins et al., 1983). It remains to be explained why the unreduced molecule shifts from 30 to 24 kD in SDS-PAGE with a minimal loss of molecular mass. It is possible that this is because of a conformation-dependent anomalous migration in SDS-PAGE.

It was recently reported that PDGF forms of 35–40 kD containing the retention sequence associate with the cell surface (La Rochelle et al., 1991, Raines and Ross, 1992); COOH-terminal proteolysis resulted in release of 30-kD PDGF. Thus, at least three forms of PDGF-BB can be formed, a 24-kD form retained intracellularly and degraded in lysosomes, a 40-kD cell surface-associated form, and a secreted 30-kD form. The final ratio of the various forms is thus likely to be dependent upon the activity of the different proteases involved in the processing. The presence of structurally different forms which differ in their subcellular compartmentalization indicates a complex regulation of PDGF-BB biosynthesis and processing. A regulatory role of proteases in the formation of secreted versus cell-bound forms has also been indicated in the biosynthesis of transmembrane growth factors like TGF-α and CSF-1 (Pandiella and Massague, 1991; Stein and Rettenmeier, 1991).

The presence of end products of PDGF-BB which differ in structure as well as compartmentalization and varies between different cell types raises questions concerning the functional properties of the two forms. The purified secreted form, that appears to be structurally identical to the well-characterized recombinant PDGF-BB (Östman et al., 1989), is mitogenically active (data not shown). It is not known whether the cell-associated 24-kD form is biologically active or if it has different functional properties compared with the 30-kD form. Several investigators champion the idea that transformation by the sis-product is mediated by an intracellular PDGF receptor activation (Keating and Williams, 1988; Huang and Huang, 1988; Bejcek et al., 1989) and there is compelling evidence that the PDGF receptor protein-tyrosine kinase is activated in an intracellular compartment in sis-transformed cells. Since receptor autophosphorylation occurs already in the ER (Keating and Williams, 1988), it is unlikely that the ligand involved is identical to the 24-kD form of PDGF-BB, which appears to be generated in the Golgi compartment according to the present study. Moreover, it is unlikely that the differences in transforming properties of the A- and B-chain of PDGF (Beckmann et al., 1988; Bywater et al., 1988) are due to differences in compartmentalization since it has recently been shown, by analyzing the transforming potential of PDGF A/B-chimeras, that high transformation potential correlated with the ability to activate both types of PDGF receptors and not with the formation of predominantly cell-associated forms (La Rochelle et al., 1990).

Thus, an important first aim for future studies will be to determine if the 24-kD form has biological activity, and to explore the possibility that it interacts with receptors of other types or in other compartments than the 30-kD form, and thus mediates different signals. Another goal for further studies will be to characterize the proteases involved in the formation of the intracellular and secreted forms and to study the regulation of these enzymes.

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