Modulation of the Transport of a Lysosomal Enzyme by PDGF

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Abstract. The major excreted protein (MEP) of transformed mouse fibroblasts is the lysosomal protease, cathepsin L. MEP is also secreted by untransformed mouse cells in response to growth factors and tumor promoters, and is thought to play a role in cell growth and transformation. To determine the relationship between MEP synthesis and MEP secretion, we have examined these events in PDGF-treated NIH 3T3 cells. PDGF enhanced MEP synthesis and caused the diversion of MEP from the lysosomal delivery pathway to a secretory pathway. These two effects were found to be regulated independently at various times after growth factor addition. Short PDGF treatments (0.5 or 1 h) resulted in quantitative secretion of MEP although synthesis was near the control level. High levels of both synthesis and secretion occurred between 2 and 14 h of PDGF treatment. Between 18 and 30 h, the amount of secreted MEP returned to the low control level even though synthesis remained elevated. The secretion was specific for MEP; other lysosomal enzymes were not found in the media from PDGF-treated cells. PDGF-induced secretion of MEP was inhibited 84% by cycloheximide, suggesting that protein synthesis is required to elicit this effect. PDGF also caused a time-dependent increase in mannose 6-phosphate (Man-6-P) receptor-mediated endocytosis. These data support a model in which PDGF alters the distribution of Man-6-P receptors such that the Golgi concentration of receptors becomes limiting, thereby causing the selective secretion of the low affinity ligand, MEP.

VIRAL transformation of fibroblasts causes major qualitative and quantitative alterations in protein synthesis and degradation that are responsible for the increased growth rate observed in transformed cells. Enhanced secretion of a number of lysosomal hydrolases has been observed in response to transforming and growth-promoting agents, and the secreted enzymes are thought to function in the processes of normal and tumor cell growth and tumor metastasis (2, 9, 14, 32, 34, 42, 43, 46).

An example of a lysosomal enzyme that may play an extracellular role during cell growth is the major excreted protein (MEP)[1] of transformed mouse NIH 3T3 fibroblasts, which has been identified as cathepsin L (7, 14, 15, 26, 27, 35, 44). This cysteine protease is localized in lysosomes in resting, untransformed NIH 3T3 cells (11, 16). However, in rapidly growing transformed cells, MEP is synthesized in large quantities and secreted (11, 17, 18). High levels of MEP are also secreted by untransformed cells in response to growth promoting agents such as PDGF and epidermal growth factor or tumor-promoting phorbol esters (3, 10, 13, 18, 41). Although direct evidence is lacking, these results suggest that secreted MEP plays some role in the growth of normal and transformed cells.

In resting NIH 3T3 cells, MEP is synthesized as a 39,000-M_r precursor that is processed intracellularly in two steps to a 20,000-M_r mature lysosomal form (16). It is the precursor form of MEP that is secreted in response to growth-promoting agents. The 39,000-M_r form of MEP contains mannose 6-phosphate (Man-6-P), the characteristic marker of most lysosomal hydrolases, and binds to Man-6-P receptors (38), the receptors responsible for efficient delivery of most lysosomal enzymes to lysosomes.

MEP made by resting cells appears to follow the same intracellular route as do other lysosomal enzymes (for review, see references 6, 12, 21, 31, 37, 47). Lysosomal enzymes are synthesized in the endoplasmic reticulum, and then transported to the Golgi apparatus where their oligosaccharide side chains are modified to contain Man-6-P. The enzymes are segregated from other glycoproteins upon binding to Man-6-P receptors present in the trans Golgi apparatus. The enzyme-receptor complexes are transported to a prelysosomal compartment where they dissociate, and the enzymes are delivered to lysosomes, while the receptors are recycled. The 270,000-M_r cation-independent Man-6-P receptor is thought to be primarily responsible for normal transport and delivery of lysosomal enzymes in most cells and tissues. However, the discovery of a second Man-6-P receptor, the 46,000-M_r cation-dependent receptor (20), suggests that at least one other system for lysosomal enzyme delivery may exist.

The diversion of MEP from the normal lysosomal delivery pathway in rapidly growing cells, even though it possesses

1. *Abbreviations used in this paper*: Man-6-P, mannose 6-phosphate; MEP, major excreted protein.

the Man-6-P marker, suggests that this transport system can be regulated. We have been studying the mechanisms responsible for enhanced secretion of MEP by transformed cells and growth factor–treated untransformed cells. In a previous report, we showed that secretion of MEP by a virally transformed NIH 3T3 cell line is specific (i.e., other lysosomal enzymes are not secreted), and that MEP synthesized by normal and transformed cells has a much lower affinity for the cation-independent Man-6-P receptor than do other lysosomal enzymes (11).

To further characterize the relationship between MEP synthesis and secretion, we have examined these phenomena in growth factor–treated, untransformed fibroblasts. This study characterizes the time-dependent increases in both synthesis and secretion of MEP and shows that these two processes are regulated independently. We also present evidence that increased secretion of MEP is a result of a growth factor–induced alteration in the lysosomal protein transport system.

Materials and Methods

Materials

Recombinant platelet-derived growth factor (c-sis) was obtained from Amgen Biologicals (Thousand Oaks, CA). Immunoprecipitin (a 10% cell suspension of fixed, protein A–containing *Staphylococcus aureus* cells) was from Bethesda Research Laboratories (Gaithersburg, MD). [^{35}S]Methionine (Tran^{35}S-label) was from ICN Radiochemicals (Irvine, CA). Antibodies directed against MEP (MP-1) and the cation-independent Man-6-P receptor (GS-6) were raised in New Zealand White rabbits as previously described (39). Cation-independent Man-6-P receptor was purified from bovine liver (38). Antisera against proliferin (23) and mitogen-regulated protein (29) were gifts from S. J. Lee and D. Nathans of Johns Hopkins University and Marit Nilsen-Hamilton and Richard Hamilton of the University of Iowa, respectively. β-Galactosidase was purified from bovine testes as previously described (8), and radio-iodinated using Bolton-Hunter reagent (39) that was purchased from ICN Radiochemicals.

Cell Culture and Growth Factor Treatments

NIH 3T3 mouse fibroblasts were routinely grown in DME, supplemented with 10% calf serum (Gibco Laboratories, Grand Island, NY), at 37°C in a humidified atmosphere of 5% CO_2. Density-arrested cells were obtained by plating cells in 24-well cluster plates (Costar, Cambridge, MA) or 60-mm plates (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) in DME with 10% calf serum, and growing them for 5 d without any change of the medium. Arrested growth was verified by [^{3}H]thymidine incorporation assays, as previously described (28).

The density-arrested cells were treated with PDGF (final concentration, 15 ng/ml) in fresh serum-free DME containing 10 ng/ml transferrin (Sigma Chemical Company, St. Louis, MO) and 4 nM sodium selenite (Gibco Laboratories). Times and other details of the growth factor treatments are described in the figure legends. The experiments were standardized according to the number of cells present at the beginning of the treatments. In most cases, this number remained constant throughout the course of the experiment. The exception was a slight loss of untreated cells during the labeling and chase periods in serum-free medium. This problem has since been solved by the addition of 1 mg/ml BSA to the labeling medium. There was no loss of PDGF-treated cells either in the absence or presence of BSA.

[^{35}S]Methionine-labeling and Immunoprecipitation of MEP

Cells in 24-well plates were labeled with [^{35}S]methionine (100–200 μCi/ml) in 0.5 ml serum-free DME containing 3 nM unlabeled methionine, 10 ng/ml transferrin, and 4 nM sodium selenite, for the times indicated in the figure legends. For pulse-chase studies, the label was "chased" with 200 nM unlabeled methionine. At the end of the labeling periods, medium was collected and cells were washed once with buffer A (10 mM sodium phosphate, pH 7.0, 0.125 M NaCl), and then lysed with 0.5 ml 1% SDS. Samples were stored at −20°C until use.

MEP was immunoprecipitated from 100 μl of medium or cell lysate with 3 μl of MEP antiserum, as previously described (11). Immunoprecipitin was used to collect the antibody-antigen complexes. Immunoprecipitates were analyzed by SDS-PAGE on gels containing a total acrylamide concentration of 12.5% (22). Gels were subjected to fluorography using En^{3}Hance (DuPont Co., Wilmington, DE/New England Nuclear, Boston, MA). Where indicated, fluorographs were scanned with a video densitometer (No. 620; Bio-Rad Laboratories, Cambridge, MA).

Isolation of ^{35}S-Labeled Man-6-P–containing Proteins from Media

Cation-independent Man-6-P receptor, immobilized on Immunoprecipitin, was used to isolate Man-6-P–containing proteins from the culture media of growth factor–treated cells. Immobilized receptor was prepared as previously described (11). Aliquots of culture media (100 μl) from biosynthetically labeled cells were incubated with saturating amounts of immobilized receptor to allow binding of Man-6-P–containing proteins. Incubations were carried out at 4°C for 60 min in buffer B (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and 0.02% NaN_3). At the end of the incubation, the samples were centrifuged and the pellets were washed 3 times with buffer B containing 1 mg/ml BSA and once with 0.1 M Tris, pH 6.8. Specifically bound proteins were released from the receptor with 10 mM Man-6-P (in 0.1 M Tris, pH 6.8). The Man-6-P releasable proteins were subjected to electrophoresis as described above.

Uptake of ^{125}I-β-Galactosidase by NIH 3T3 Cells

High-affinity forms of ^{125}I-β-galactosidase were isolated using Man-6-P receptor immobilized on Immunoprecipitin. Immobilized receptor was prepared as described previously (11). ^{125}I-β-Galactosidase was incubated with immobilized receptor in TBS (50 mM Tris, pH 7.2, 150 mM NaCl) containing 1 mg/ml BSA for 45 min at 4°C. The Immunoprecipitin was then washed five times with TBS containing 1 mg/ml BSA. High affinity β-galactosidase was eluted with 5 mM Man-6-P in the same buffer. The preparation was dialyzed against 50 mM sodium phosphate, pH 7.2, 150 mM NaCl before use in the uptake experiments.

Cells in 60-mm plates were pretreated with 15 ng/ml PDGF in serum-free DME containing 10 ng/ml transferrin, 4 nM sodium selenite, and 1 mg/ml BSA for various times. The cells were then chilled on ice and treated for 15 min with ice-cold 2 mM Man-6-P to remove any endogenous cell surface ligands. Cells were washed twice with Hepes-buffered DME (50 mM Hepes, pH 7.2) containing 1 mg/ml BSA and twice with Hepes-buffered DME alone. Prewarmed (37°C) uptake medium (serum-free DME plus 1 mg/ml BSA), containing 100,000 cpm high-uptake ^{125}I-β-galactosidase/plate, was added and the uptake was carried out for 30 min at 37°C. The cells were then returned to the ice bath, the medium was removed, and cells were washed once with Hepes-buffered DME plus 1 mg/ml BSA, twice with Hepes-buffered DME, and once with Hepes-buffered saline. Cells were lysed with 1 ml of 1% sodium deoxycholate and radioactivity in the lysates was determined with a counter (Gamma 4000; Beckman Instruments, Palo Alto, CA). Protein concentration of the lysates was assayed according to the method of Lowry (25). Specific uptake via the Man-6-P receptor was determined by subtracting uptake values obtained in the presence of 5 mM Man-6-P from values obtained in the absence of Man-6-P for each pretreatment time.

Results

Effect of PDGF on Secretion of Proteins by NIH 3T3 Cells

When NIH 3T3 fibroblasts are treated with PDGF, there are both qualitative and quantitative changes in secreted proteins. Previous studies have demonstrated that PDGF induces both synthesis and secretion of MEP (13, 41), but the actual level of secretion relative to the total amount synthesized has not been determined. Fig. 1 shows that PDGF treatment of NIH 3T3 cells results in secretion of most of the MEP made by these cells. The effects of PDGF on total protein secretion are shown on the left, and MEP secretion is illustrated on the right. PDGF caused enhanced secretion of

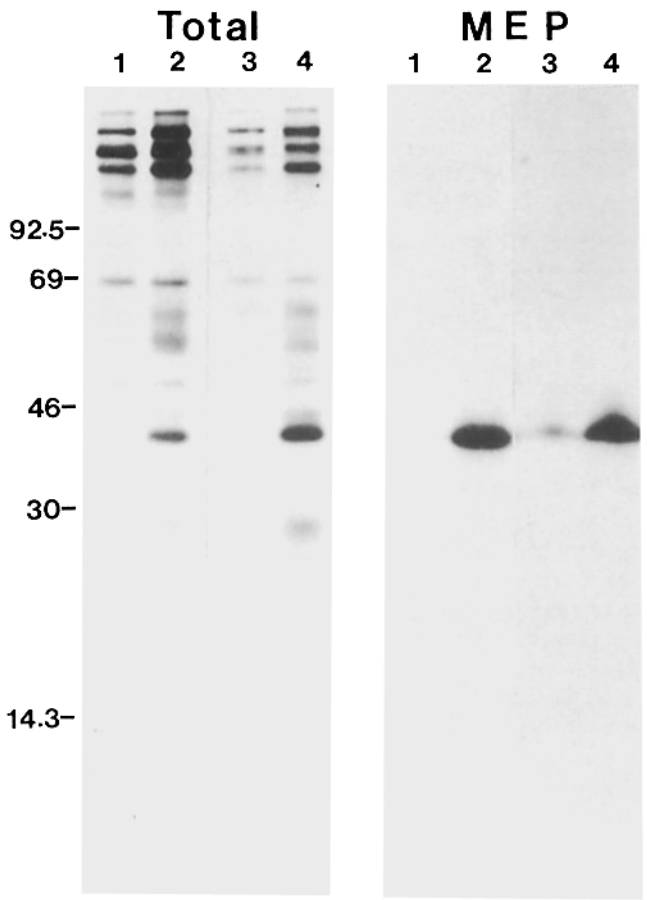

Figure 1. Effects of PDGF on MEP and total protein secretion by NIH 3T3 cells. Density-arrested NIH-3T3 cells were labeled for 18 h with 200 μCi/ml [^{35}S]methionine in serum-free DME with no additions (lane *1*), or in the presence of 15 ng/ml PDGF (lane *2*), 10 mM NH_4Cl (lane *3*), or PDGF plus NH_4Cl (lane *4*). Shown is a fluorograph of SDS-polyacrylamide gels of the total proteins present in 10 μl media (*left*) and immunoprecipitates of MEP (*right*) from 100 μl media.

a number of proteins, but the most dramatic effect was on MEP. Since treatment of cells in culture with NH_4Cl causes secretion of newly synthesized lysosomal hydrolases (19), the percent of the total MEP that was secreted was estimated by comparing the amount of MEP secreted in the absence of NH_4Cl (lanes *1* and *2*) to that secreted by the NH_4Cl-treated cells (lanes *3* and *4*). Control cells secreted very little (<10%) newly synthesized MEP, whereas in PDGF-treated cells most of the MEP was secreted. Also, by comparing the amount of MEP secreted by NH_4Cl-treated control cells (lane *3*) and PDGF-treated cells (lane *4*), it is evident that PDGF caused an ~10-fold increase in MEP synthesis as well as increasing MEP secretion.

Time Course for PDGF-Mediated Effects on MEP Synthesis and Secretion

To compare time-dependent effects of PDGF on MEP secretion to effects on MEP synthesis, we determined the amount of time required for PDGF to affect MEP synthesis in our tissue culture system. As shown in Fig. 2, PDGF treatments of <1 h had little effect on the amount of MEP synthesized by NIH 3T3 cells. However, after a 2-h exposure to PDGF, MEP synthesis was seven times higher than in control cells (as determined by densitometry), and leveled off at an 8–9-fold increase between 3 and 4 h after PDGF addition. The effect of PDGF on MEP synthesis did not require the continued presence of the growth factor; treatment of the cells with PDGF for as little as 30 min yielded the same increases in MEP synthesis at later times as those seen when PDGF was present throughout the entire experiment (data not shown).

The level of MEP secretion by the cells after various times of incubation with PDGF was examined next. As a means of estimating the total amount of MEP synthesized during the labeling period, we took advantage of the effects of NH_4Cl on secretion of Man-6-P–containing lysosomal enzymes, as described above. The amount of labeled MEP secreted by NH_4Cl-treated cells represents the total amount of MEP made during the labeling period. The amount of MEP secreted in the absence of the amine represents the portion of the total which was secreted in response to the growth factor. The validity of these assumptions has been verified by pulse-chase studies (data not shown).

As shown in Fig. 3, after short treatments (0.5 or 1 h) with PDGF, the amount of MEP synthesized (+NH_4Cl lanes) remained near the control level. However, in contrast to the control, nearly all of the MEP was secreted (compare −NH_4Cl lanes with +NH_4Cl lanes). After 2 h of growth factor treatment, the cells synthesized a larger amount of MEP, which continued to be made after all of the other pretreatment times examined (+NH_4Cl lanes). Although the level of MEP synthesis remained high from 2 to 30 h after PDGF addition, an elevated level of secretion was observed only until 18 h after the growth factor was added. From 18 to 30 h following PDGF addition, MEP secretion was dramatically reduced (−NH_4Cl lanes) even though the amount synthesized (+NH_4Cl lanes) remained high. Thus, MEP

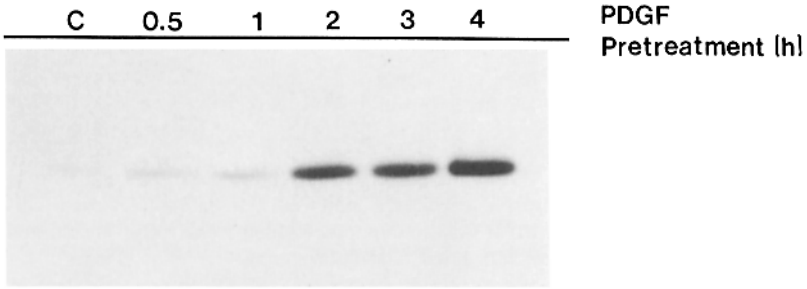

Figure 2. Time course for PDGF induction of MEP synthesis. Density-arrested cells were treated with 15 ng/ml PDGF for the indicated times. Cells were then labeled for 30 min with 150 μCi/ml [^{35}S]methionine. At the end of the labeling, the medium was removed and cells were washed twice with buffer A containing 1 mg/ml BSA, and then lysed with 500 μl 1% SDS. MEP was immunoprecipitated from 100 μl of the cell lysate, and the immunoprecipitates were subjected to electrophoresis and fluorography.

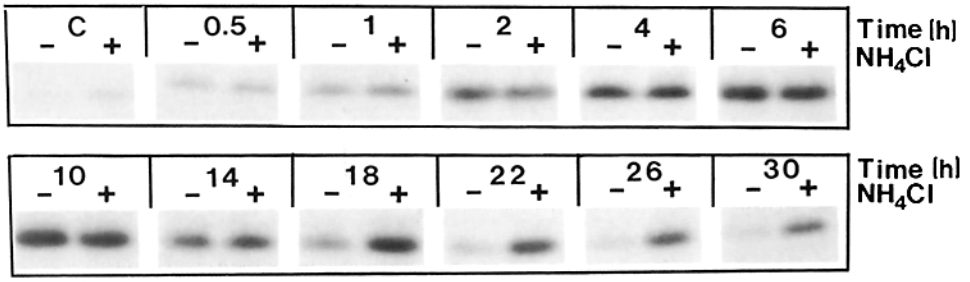

Figure 3. Time-dependent effects of PDGF on MEP secretion. Density-arrested cells were pretreated with 15 ng/ml PDGF for the indicated times. Cells were then labeled with 150 μCi/ml [^{35}S]methionine for 30 min, after which the label was "chased" for 5 h with excess unlabeled methionine in the absence or presence of 10 mM NH_4Cl, as indicated. Media samples were collected, and MEP was immunoprecipitated from 100 μl of each sample. The immunoprecipitates were analyzed by electrophoresis and fluorography.

synthesis and secretion were regulated independently by PDGF. Similar results were obtained when cells were treated with fibroblast growth factor (data not shown).

Low Level of MEP Secretion after Prolonged PDGF Treatment Is not Because of Secretion-Recapture

It was possible that the low level of MEP secretion observed after long PDGF treatments (18–30 h) was because of secretion of the MEP during the early portion of the experimental chase period, with subsequent internalization via cell surface Man-6-P receptors at a later time. If this were the case, addition of Man-6-P to the chase medium should inhibit recapture. As shown in Fig. 4, we found that Man-6-P had no effect on the level of MEP secreted by cells pretreated for 26 h with PDGF, indicating that MEP was not secreted and then recaptured during the course of the experiment. This finding was confirmed by pulse-chase studies in which secretion of MEP was monitored at various times during the chase (not shown). At no time were high levels of MEP found to be secreted under these conditions. Thus, the apparent adaptation of the cells to prolonged treatment with PDGF is related to a change in intracellular trafficking of MEP rather than to an alteration in endocytosis of secreted MEP.

Specificity of MEP Secretion by PDGF-Treated Cells

One possibility that could explain the enhanced secretion of MEP by PDGF-treated cells is that PDGF causes a general change in the lysosomal enzyme transport pathway, such that proteins that are normally delivered to lysosomes are secreted. If PDGF has such an effect on the transport of lysosomal enzymes, other lysosomal enzymes should also be secreted by PDGF-treated cells. Alternatively, the effects of PDGF on secretion of lysosomal enzymes could be selective for MEP. To distinguish between these two possibilities, the media from PDGF-treated cells were examined for other lysosomal, Man-6-P–containing proteins.

Lysosomal enzymes that were secreted by biosynthetically labeled PDGF-treated cells were isolated with immobilized Man-6-P receptor. It was assumed that since MEP secreted by these cells contains Man-6-P, other secreted lysosomal enzymes would also contain the Man-6-P marker. Upon comparing Man-6-P–containing proteins that were secreted in the absence of NH_4Cl to those secreted in the presence of NH_4Cl, we could determine if secretion of other lysosomal enzymes was affected by PDGF. Fig. 5 shows that MEP was the only detectable Man-6-P–containing protein secreted by PDGF-treated cells; other major lysosomal proteins were not secreted in the absence of NH_4Cl. Also, as indicated by the lanes corresponding to the NH_4Cl-treated cells, the synthesis of most of the other lysosomal enzymes was not affected by PDGF. The exception was a 27,000-M_r protein whose synthesis (+NH_4Cl lane), but not secretion (−NH_4Cl lane), was induced after 6 h of PDGF treatment. This protein was identified as proliferin (23), also called mitogen-regulated protein (29), using two different antisera specific for that protein (data not shown).

PDGF-induced MEP Secretion Requires Protein Synthesis

As a means of further defining the requirements for secretion of MEP upon addition of PDGF to density-arrested fibroblasts, we examined the effect of protein synthesis inhibitors on this secretion. For the experiments described in Table I, cycloheximide and actinomycin D were present only during the chase period, so that the amount of MEP labeled during the pulse was the same as in cells that were not treated with the inhibitors. PDGF-induced MEP secretion was dramatically inhibited (84% decrease) in the presence of cycloheximide. Total secretion of ^{35}S-labeled proteins was decreased by only 20% under these conditions, although >90% of all protein synthesis was inhibited during the chase period (data

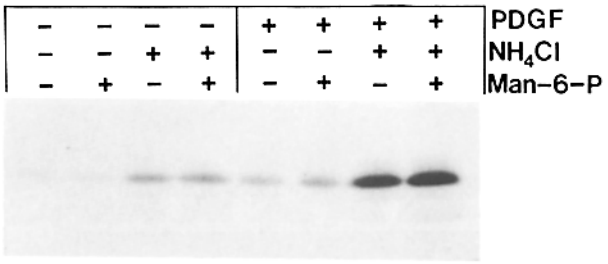

Figure 4. Low MEP secretion after long-term PDGF treatment is not because of secretion-recapture. NIH 3T3 cells were pretreated for 26 h with or without 15 ng/ml PDGF, and labeled as described for Fig. 3. During the chase period, 10 mM Man-6-P and 10 mM NH_4Cl were present where indicated. Media samples were collected and MEP was immunoprecipitated from 100 μl media and analyzed by SDS-PAGE and fluorography.

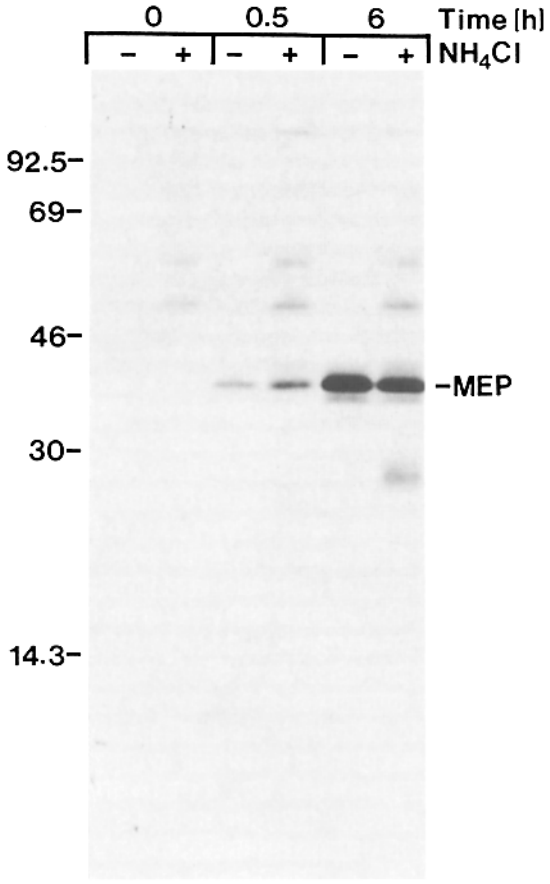

Figure 5. Specificity of MEP secretion in response to PDGF. Media samples from untreated cells or cells pretreated with PDGF for 0.5 or 6 h, labeled as described for the experiment in Fig. 3, were incubated with immobilized Man-6-P receptor in order to isolate all secreted Man-6-P-containing lysosomal enzymes. Proteins that specifically bound to the receptor were released with 10 mM Man-6-P, and subjected to electrophoresis and fluorography. The difference in the amount of MEP present in the $+NH_4Cl$ lanes for the no addition and 0.5 h pretreatment times is attributed to a loss of some of the untreated cells during the course of the experiment.

not shown). This indicates that cycloheximide did not have an adverse effect on general protein secretion, but that the inhibition of MEP secretion was relatively specific. Similar inhibition of MEP secretion was observed when puromycin (10 μg/ml) was used to block protein synthesis. Examination of cell lysates from the cycloheximide-treated cells revealed no accumulation of the precursor form of MEP in these cells (data not shown). The site at which degradation occurred was not determined. These results suggest that secretion of MEP is dependent on the synthesis of a protein(s) during the experimental chase period. The effect of this protein(s) on MEP secretion is apparently short lived since it is abruptly lost upon addition of cycloheximide or puromycin.

One explanation for the inhibition of MEP secretion by cycloheximide is that high levels of MEP synthesized during the experimental chase period are responsible for PDGF-induced secretion of the labeled MEP. (This could result from saturation of the transport system for MEP as suggested for virally transformed cells [11]). To investigate this possibility, synthesis of MEP mRNA during the chase was inhibited with actinomycin D. We found that addition of actinomycin D to the PDGF-treated cells at the end of the labeling period resulted in a >90% inhibition of MEP synthesis and 40% inhibition of total protein synthesis during the 5-h chase (data not shown). However, in contrast to the dramatic inhibition caused by cycloheximide, PDGF-induced MEP secretion was only slightly affected by actinomycin D (35% decrease). These results indicate that the effect is not because of increased synthesis of MEP but instead to some constitutively synthesized factor whose message is long-lived relative to that of MEP.

Table I. Effect of Cycloheximide and Actinomycin D on MEP Secretion

Treatment	Percent secretion*
None	⩽15
PDGF	103 ± 23
PDGF + cycloheximide	19 ± 3
PDGF + actinomycin D	68 ± 13

* Percent secretion refers to the amount of MEP secreted in the absence of NH_4Cl relative to the amount secreted in the presence of 10 mM NH_4Cl for each condition.

Density-arrested cells were pretreated with 15 ng/ml PDGF for 1 h and then pulse-labeled with [^{35}S]methionine as described for Fig. 3. The label was then "chased" for 5 h in the absence or presence of 10 mM NH_4Cl and in the absence or presence of 10 μg/ml cycloheximide or actinomycin D. MEP was immunoprecipitated from the media samples and analyzed by SDS-PAGE. Values were obtained by densitometric scanning of fluorographs, and are expressed as the mean ± 1 SD obtained from three separate experiments.

Effect of PDGF on Man-6-P Receptor-mediated Endocytosis

PDGF-induced altered trafficking of MEP, a protein that is normally transported by Man-6-P receptors, suggests that the transport system itself may be affected in PDGF-treated cells. One measure of Man-6-P receptor function is the endocytosis of Man-6-P-containing ligands. We used [^{125}I]β-galactosidase as a tool to investigate possible effects of PDGF

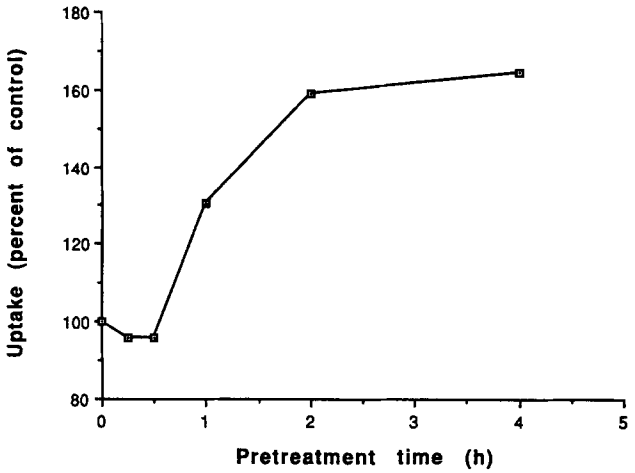

Figure 6. Effect of PDGF on [^{125}I]β-galactosidase uptake by NIH 3T3 cells. Density-arrested NIH 3T3 cells were either left untreated or were pretreated with PDGF (15 ng/ml) for 0.25, 0.5, 1, 2, or 4 h and then tested for the ability to uptake [^{125}I]β-galactosidase via Man-6-P receptors. The data are expressed as the percent of the control uptake that is the amount of [^{125}I]β-galactosidase taken up by untreated cells. Shown are the mean values obtained from two separate determinations.

on Man-6-P receptor-mediated endocytosis. Density-arrested cells were pretreated with PDGF for various times and then assayed for their ability to uptake [^{125}I]β-galactosidase. As shown in Fig. 6, after a 30-min lag, PDGF treatment resulted in a time-dependent increase in [^{125}I]β-galactosidase uptake that reached a plateau at 165% of the control value by 2 h. Therefore, PDGF affected Man-6-P receptor function within a time frame similar to that of the PDGF-mediated increase in MEP secretion.

Discussion

To define mechanisms responsible for growth-related changes in the transport of lysosomal enzymes, and to identify possible roles of secreted lysosomal hydrolases in growth and metastasis, we have been examining lysosomal protein synthesis and trafficking in NIH 3T3 fibroblasts transformed with an oncogenic virus or treated with growth factors. Previous studies have shown that increased amounts of MEP are synthesized and secreted by mouse fibroblasts upon viral transformation or growth factor treatment, and it has been proposed that MEP overproduction alone is sufficient to account for the enhanced secretion (11, 44). The present investigation examines the time dependency of PDGF-induced synthesis and secretion of MEP by untransformed NIH 3T3 cells, and provides evidence that MEP secretion is not a direct result of its increased synthesis as previously suggested.

PDGF treatment of the cells resulted in time-dependent increases in both MEP synthesis and MEP secretion. MEP synthesis was induced between 1 and 2 h after PDGF addition, and remained high for at least 30 h (Figs. 2 and 3). This is consistent with a previous study by Scher et al. (41), which demonstrated that in BALB/c 3T3 cells PDGF enhanced MEP synthesis within 40 min after growth factor addition, with a high level of synthesis continuing through 24 h. Increased synthesis of MEP in response to PDGF and viral transformation has been attributed to increased levels of MEP messenger RNA (13, 18).

PDGF-enhanced MEP secretion was found to be regulated independently of MEP synthesis. After short PDGF treatments ($\leq$1 h), when MEP synthesis was still near control levels, newly synthesized MEP was secreted. After prolonged incubation with PDGF (>18 h) the cells secreted progressively less MEP, despite the continued high level of MEP synthesis. These results indicate that signals generated by PDGF treatment regulate intracellular trafficking of MEP and that this regulation is different from that responsible for the effects on MEP synthesis.

The PDGF-induced alteration in MEP trafficking was found to be specific, in that secretion of other lysosomal proteins was not significantly affected (Fig. 5). While this suggests the possibility that altered trafficking of MEP results from specific PDGF-induced changes in MEP, no such changes have been found. MEP made by PDGF-treated NIH 3T3 cells has the same affinity for the cation-independent Man-6-P receptor as MEP made by quiescent cells (our unpublished results). Also, the MEP made by resting and growth factor-treated NIH cells, as well as MEP made by transformed NIH cells, display similar peptide maps (16, 18). Thus, it appears that secretion of MEP results from PDGF-induced alterations in the lysosomal protein transport system rather than from changes in MEP itself. Lysosomal delivery of MEP in quiescent NIH cells is mediated by the cation independent Man-6-P receptor, as demonstrated by the inhibition of lysosomal delivery of MEP when antibodies to this receptor are added to the culture medium (our unpublished data). Thus, the most likely target for the PDGF effect is the cation-independent Man-6-P receptor.

Signals generated by PDGF could act by causing a reduction in the total concentration of functional cation-independent receptors within the cell. Because of the intrinsic low affinity of MEP for the receptor, such a condition would be expected to cause selective secretion of MEP by "default," via the constitutive secretory pathway (11). However, preferential secretion of MEP was not observed when cation-independent Man-6-P receptors were progressively depleted by treatment of cells with increasing concentrations of NH_4Cl or receptor antibodies (our unpublished data); MEP secretion paralleled secretion of other Man-6-P-containing proteins under those conditions. Also, a direct assay of Man-6-P receptors revealed no differences in receptor levels between untreated and PDGF-treated cells (our unpublished data). These results indicate that a simple reduction in the total levels of cation-independent receptors is not responsible for the effects on MEP transport observed in PDGF-treated cells.

An alteration in the interaction of the receptor with MEP might also be caused by changes in the distribution or binding properties of the receptor, or in the rate of transit of the receptor or MEP through the cell. Fig. 6 shows that PDGF does affect Man-6-P receptor function, in that endocytosis via this receptor is greatly increased in PDGF-treated cells. Such an effect by PDGF is indicative of an alteration in receptor distribution or cycling rate. Future studies will determine whether the enhanced uptake is because of more receptors present at the cell surface or an increased rate of endocytosis of cell surface receptors. Increased levels of cation-independent Man-6-P receptors at the cell surface in response to insulin (1, 4, 5, 24, 30, 48), insulin-like growth factor I, insulin-like growth factor II, and epidermal growth factor (1) have been reported for other cell lines. Therefore, it is likely that the increased uptake we observed is because of a similar redistribution of receptors. Our data support a model in which PDGF alters the distribution of Man-6-P receptors, such that the Golgi concentration of receptors becomes limiting; the receptors become saturated with high affinity lysosomal enzymes, and low affinity MEP is secreted instead of being transported to lysosomes.

While a mechanism involving modulation of cation-independent Man-6-P receptor function is favored, we cannot rule out the possibility that the cation-dependent receptor, or some as yet unknown receptor or binding protein, is involved in PDGF-induced MEP secretion. Since lysosomal delivery of MEP in these cells is normally mediated by the cation-independent receptor, other proteins involved in MEP secretion would most likely act by preventing the interaction of MEP with the cation-independent Man-6-P receptor.

The finding that cycloheximide dramatically inhibits PDGF-induced MEP secretion (Table I) suggests that protein synthesis is required to produce this effect. It appears that the protein(s) involved is rapidly turned over, since addition of cycloheximide 6 h after the PDGF also blocked subsequent MEP secretion (our unpublished observations). Actinomycin D, which inhibits MEP synthesis by >90%, had only a slight effect on MEP secretion, to the same extent as the

effect on total protein synthesis, indicating that the inhibition was due to a decrease in the synthesis of some protein other than MEP. One explanation for the cycloheximide-mediated inhibition of MEP secretion in PDGF treated cells is that cycloheximide decreases the synthesis of other lysosomal enzymes, resulting in a decrease in the occupancy of Golgi Man-6-P receptors by other ligands, thereby allowing the binding and lysosomal delivery of MEP. An alternate explanation is that in response to PDGF, some short-lived protein, whose synthesis is inhibited by cycloheximide, directly participates in the diversion of MEP from the lysosomal delivery pathway. If this is the case, the identification and characterization of such a protein will be critical to further studies on the mechanisms of regulated MEP secretion. PDGF-induced MEP mRNA synthesis also requires protein synthesis (13, 36). Whether the same or different proteins are responsible for these two events remains to be determined.

Although the evidence accumulated thus far is correlative, it does appear that there is a link between cell growth and lysosomal enzyme secretion. The specificity and time dependency of PDGF-induced MEP secretion suggests that this is a highly regulated process. The role of secreted MEP in the growth process is unknown, but some conclusions can be drawn from the observations described above. First, MEP secretion is stimulated by growth factors such as PDGF and fibroblast growth factor, which render cells competent (33) to enter into mitosis, suggesting that MEP secretion is related to the cells' acquisition of "competence." Furthermore, MEP secretion was maximal after 6–10 h, whereas DNA synthesis was not observed until 12 h after PDGF addition and continued to rise through 18 h (our unpublished results), at which time MEP secretion was decreased. Therefore, MEP may be needed to prepare the cells or extracellular milieu for mitosis. Since MEP is a cysteine protease, it is possible that secreted MEP hydrolyzes extracellular matrix components (14) or proteins on the cell surface. Indeed, purified MEP has been shown to degrade collagen, fibronectin, and laminin in vitro (14). Further studies investigating these and other possibilities should provide definitive proof of MEP's function in cell growth.

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