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Characterization of a Sheep Pituitary-Derived Growth Factor for Rat and Human Mammary Tumor Cells

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A growth factor for rat and human mammary tumor cells (MTGF-Pit) was isolated from lyophilized powders of whole sheep pituitaries by a rapid four-step procedure utilizing acetic acid extraction, heating at 93°C, and sequential chromatography in 0.10 M acetic acid on sulphopropyl Sephadex and Sephadex G-50. From 10 g of pituitary powder, 8–10-mg amounts of MTGF-Pit were isolated. By 8 M urea, 0.1% SDS-12.5% polyacrylamide gel electrophoresis analysis followed by Coomassie blue staining, this preparation was shown to be one major stained band. When assayed for growth effects on cells maintained in serum-free medium, 5.1–19.2 nM MTGF-Pit half replaced the growth of MTW9/PL rat and MCF-7 and T-47D human mammary tumor cells in response to 2% to 10% serum. MTGF-Pit shows mitogenic activity toward normal human diploid fibroblasts only at concentrations in excess of 2.5×10^{-4} M, while rat fibroblasts are unresponsive even at this high concentration. From data available, we conclude that a mitogenic activity for epithelial-type mammary cells has been isolated, and this growth factor appears to be a previously undetected acid- and heat-stable activity that is highly abundant (estimated at 0.16% or more of the total dry weight of the pituitary powder). The isolated ovine MTGF-Pit (3,900 ± 200 daltons) does not share the molecular weight of native prolactin (24,000 daltons), "cleaved" prolactin (16,000 daltons), or growth hormone (22,000 daltons), and by all tests applied cannot be replaced with other known hormones and purified growth factors. We conclude a potent new mammary tumor cell mitogenic activity has been identified from sheep pituitaries.

Key words: human and rat mammary tumor cells, polypeptide growth factor, peptide-isolation methods, sheep pituitary gland, estrogen-responsive cell growth, prolactin, growth hormone

The growth of both rat and human mammary tumor cells in host animals has been confirmed to be pituitary factor responsive [1–9]. Using the MTW9/PL rat mammary tumor cells developed from the MTW9A hormone-responsive tumor of

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W/Fu rats [6], we have shown previously [10] that these cells form 1.8–7.6-fold larger tumors in estrogen-treated hosts bearing the GH3/C14 rat growth hormone (GH)- and prolactin (PRL)-secreting pituitary tumor than in estrogen-treated hosts not implanted with pituitary tumors. The possible role of pituitary origin hormones or growth factors in growth of MTW9/PL cells was further confirmed in vitro by demonstrating that MTW9/PL cell growth in culture was supported less well by serum prepared from hypophysectomized female rats than serum prepared from intact females [10].

Using human breast cancer cells, Shiu [7] and Leung and Shiu [8] have reported that the T-47D line formed estrogen-responsive tumors in athymic nude mice and that this growth was stimulated 14-fold when these animals bear a separate GH- and PRL-secreting GH3 rat pituitary tumor. Welsch et al [9] made similar observations studying the estrogen-responsive growth of the human origin MCF-7 mammary tumor cells in athymic mice bearing GH3 tumors, and McManus and Welsch [11] extended these observations showing that maintenance and growth of normal human breast tissue in athymic nude mice was also increased by the co-implantation of the GH3 pituitary tumor.

In a recent report Leung et al [12] presented preliminary evidence that a 46,000–60,000-dalton growth factor extracted from GH3 cells promoted proliferation of the T-47D human tumor cells in culture. Additionally, Hammond and Ham [13] have shown for the first time that long-term cultures of normal human breast cells can be grown in a serum-free hormonally defined medium supplemented with PRL (at 5 μg/ml) and an undefined extract of bovine pituitary gland. Since the addition of both PRL and pituitary extract were required, the data suggested that pituitary extract contained growth-supporting substances different from PRL.

From the data available with these cell lines, as well as from a broad range of studies describing pituitary effects on mammary growth in vivo [1–9], the implications were that either GH or PRL (or their combination) were directly mitogenic for both normal and malignant mammary issue. However, our previous report [10] had shown that MTW9/PL cell growth was not PRL or GH responsive in culture as assayed by methods directly measuring an increase in cell number; instead, MTW9/PL cells were responsive to another type of pituitary activity which could be extracted from either normal pituitaries [10] or from GH3/C14 rat pituitary tumors [10]. When extracted at neutral pH under nondissociating conditions, this activity on MTW9/PL cells had an apparent molecular weight of 50,000–80,000 daltons and fell within the same molecular weight range as the GH3 growth factor for T-47D cells reported by Leung et al [12]. Having obtained these results, the major problem then became determining what factors or hormones from pituitary other than PRL and GH are important in mammary tumor growth. At issue in all of the reported studies was whether the mammary cell mitogen(s) was actually within the 46,000–80,000-dalton range, or instead was a low molecular weight species associated with other components of the crude extracts. For example, we have previously identified an estrogen-inducible mammary tumor cell mitogen in extracts of rat uteri [14,15]. When prepared under nondissociating conditions, the molecular weight of this activity was estimated at 70,000 daltons [16]; when completely purified under dissociating (acid pH) conditions, the growth factor proved to be a 4,200-dalton polypeptide [17]. Similarly, both epidermal growth factor (EGF) and insulinlike growth factor I (IGF-I) were identified as 73,000-dalton growth factors or larger at neutral pH, but when

purified under acidic conditions proved to be less than 10,000 daltons [18–20]. Likewise, several forms of PRL have been reported, ranging in molecular weights from 60,000 to 16,000 daltons. The higher molecular weight forms found in tissue and plasma [21] have been only circumstantially related to mammary tissue growth in vivo, whereas the 16,000-dalton form (designated cleaved PRL) has been reported to be mitogenic for normal rat mammary gland in vivo [22,23]. In order to resolve the issue of the actual molecular weight of the pituitary origin mitogen and to establish whether native PRL or "cleaved PRL" are the only mitogenic agents identifiable from pituitary, we isolated the mammary tumor cell mitogenic activity from normal sheep pituitaries under dissociating conditions.

Using a new acetic acid extraction method, we describe here the isolation of mg quantities of a mitogenic activity for MTW9/PL, MCF-7, and T-47D mammary tumor cells. From small amounts of lyophilized powders of sheep pituitaries, a low molecular weight (ie, 3,900 daltons) mammary tumor cell growth factor (MTGF-Pit) was isolated and shown not to correspond to the known properties of native PRL (24,000 daltons), GH (22,000 daltons), or to the molecular weight of a recently described form of PRL, designated cleaved PRL (16,000 daltons). The implications of our results are that the pituitary may possess a substantial amount of a previously undetected mammary cell tumor growth factor activity with considerable biological significance.

MATERIALS AND METHODS

Cell Line Growth

The MTW9/PL cell line used in these studies was established from the estrogen- and pituitary-hormone-responsive MTW9A tumor induced in W/Fu rats by a single challenge of 3-methylcholanthrene [1]. Over a period of 6 years, the MTW9/PL cells have retained the ability to form estrogen-responsive [6], thyroid-responsive [6], and pituitary-factor-responsive [10,14] tumors in rats. Stock cultures of the MTW9/PL rat mammary tumor cells were maintained on 10-cm-diameter plastic petri tissue culture dishes (Corning Glass Works, Corning, New York) at 37°C in a humid atmosphere of 95% air and 5% CO_2. The cells were grown in Dulbecco modified Eagle medium (DME) purchased from Grand Island Biological Co. (Grand Island, NY) as the 4.5-g glucose/liter formulation, and supplemented with 2.2 g sodium bicarbonate, 2mM glutamine, 240 μg/ml penicillin G, 540 μg/ml streptomycin sulfate, 50 μg/ml ampicillin, 15 mM HEPES (pH 7.2), and 10% (vol/vol) fetal calf serum (FCS). Logarithmic growth cultures were maintained by passage of 6.0×10^5 cells per 10-cm-diameter dish twice per week. The GH3/C14 rat pituitary cells [24] used in this study were grown under conditions similar to those used for MTW9/PL cells with two exceptions. The DME medium was supplemented with 12.5% (vol/vol) horse serum (HS) and 2.5% (vol/vol) FCS, and the cells were passaged at 4.0×10^5 per 10-cm-diameter petri dish twice per week. The T-47D human mammary cells were grown under the same conditions as MTW9/PL cells with the exception that 8.0×10^5 cells were passaged per dish twice per week. The human foreskin fibroblasts, normal rat ear cartilage fibroblasts and the MCF-7 cells were grown under conditions identical to those of the MTW9/PL cells. Both the T-47D and MCF-7 cell lines were obtained from the EG and G Mason Research Institute Human Tumor Bank (Worcester, MA), and from the American Type Culture Collection (Rockville, MD).

Growth Factor Assays

The isolation of MTGF-Pit was monitored by measuring the effect of the mitogen on incorporation of methyl ^{3}H-thymidine into DNA of MTW9/PL cells in culture. A detailed description of this assay methodology has been presented elsewhere [14,17]. Protein determinations were performed by the method of Bradford [25], using bovine serum albumin as standard and reagents purchased from Bio-Rad Laboratories (Richmond, CA). The specific activities of the MTGF-Pit preparations were estimated in serum-free medium as the protein concentration required to replace half the mitogenic response of MTW9/PL cells to 10% (vol/vol) FCS. This value was designated G_{50}. Incorporation of label in response to serum-free DME only was designated C_0, while incorporation in response to 10% FCS was designated C_{10}. A unit of MTGF-Pit is that amount of mitogen required to half replace the 10% FCS-stimulated growth response ($G_{50} = [C_{10} - C_0]/2.$)

The T-47D cell assay was conducted exactly as described for the MTW9/PL cells [14, 17] with the following exceptions: a total of 10,000 cells were plated initially in 1.0 ml of medium containing 2% (vol/vol) FCS and 1.0 mg/ml bovine serum albumin. After 24 hr at 37°C, the serum-containing medium was removed and replaced with 0.9 ml of serum-free DME containing 1.0 mg/ml bovine serum albumin. Thereafter, the assay proceeded as with MTW9/PL cells. The MCF-7 cell assay was conducted as described for the MTW9/PL cells [14,17] with the following exceptions: a total of 10,000 cells were plated initially in 1.0 ml of medium containing 5% (vol/vol) FCS. After 24 hr at 37°C, the medium was changed to 0.9 ml of serum-free DME, and the assay proceeded as with MTW9/PL cells [17]. The assays for MTGF-Pit on growth of the human fibroblasts and normal rat fibroblasts were performed under conditions identical with those of the MTW9/PL cells. The mitogenic effect of MTGF-Pit on GH3/C14 cells was assayed by two methods. These differed in the times of pulse labeling after addition of growth factor. Method 1 began with plating 3.0×10^4 cells in Costar 24-well cluster plates in 1.0 ml of DME containing 2.5% (vol/vol) FCS and 2.5% HS (vol/vol). After 24 hr, the serum-containing medium was replaced with 0.9 ml of serum-free DME that contained no growth factor or serum additions (C_0), 5% (vol/vol) serum (C_{5d}), or growth factor only. After 48 hr 1.0 μCi of tritium-labeled thymidine was added for 2 hr at 37°C. The pulse labeling was stopped and incorporation into DNA determined as before [14,17]. The second assay method differed from the first in that the cells were plated in DME containing 5% (vol/vol) FCS and 5% (vol/vol) HS; after 24 hr, the serum-containing medium was replaced with serum-free medium containing an addition of 0.3% bovine serum albumin. After an additional 24 hr, growth factor (or serum) was added and the pulse labeling (2 hr) with tritium-labeled thymidine done 22 hr later as described above. The amount of label incorporated in response to no added growth factor was designated C_0. Incorporation in response to 10% serum was designated C_0. These values allowed calculation of the half-maximal growth response (G_{50}) as described above for the MTW9/PL cells.

Tissue Source

The lyophilized powder of whole sheep pituitaries (male and female mixed) was obtained from Waitaki Refrigerating Limited (Christchurch, New Zealand). Pituitaries were removed from the sheep, rinsed free of residual blood, frozen in liquid nitrogen, lyophilized, and converted to a powder by stainless steel ball mill treatment.

The powder represents 20–25% of the wet weight of the tissue and was stored at −20°C in sealed plastic bags to avoid humidity. In this form the MTGF-Pit activity is stable indefinitely.

Method of Isolation of Ovine MTGF-Pit

Lyophilized powder of sheep pituitaries was used to isolate the MTGF-Pit. The method of approach used was a four-step procedure based on our previous purification of a sheep uterine-derived mammary tumor cell growth factor [17]. The initial four steps were used as described with the single exception that the methods were applied on a 1/50 scale. The purification steps used included the sequential extraction of pituitary powder in 0.1 M acetic acid, followed by heating at 93°C for 5 min to remove impurities. The active heated supernatant from the acetic acid extract was then applied to sulphopropyl Sephadex C-25 column chromatography in 0.1 M acetic acid, and MTGF-Pit eluted with 0.3 M ammonium acetate, pH 7.2. This activity was then concentrated by lyophilization and applied to a Sephadex G-50 column equilibrated and eluted with 0.1 M acetic acid. The active fractions from Sephadex G-50 were concentrated by lyophilization, redissolved in 0.1 M acetic acid, and stored at 4°C; MTGF-Pit was >80% stable for 4 months under these conditions.

Polyacrylamide Gel Electrophoresis

The degree of homogeneity and the molecular weight of the isolated MTGF-Pit was estimated by 8 M urea, 0.1% SDS polyacrylamide gel electrophoresis (PAGE), using 12.5% acrylamide gels formed in 6 × 90-mm tubes. Electrophoresis, at pH 6.8, was conducted as described by Swank and Munkres [26] with the exception that reducing agents (β-mercaptoethanol or dithiothreitol) were omitted from the running buffers and from the sample boiling solution. After electrophoresis, the gels were fixed and stained in 0.2% Coomassie blue dye dissolved in methanol/acetic acid/water (5:1:5 vol/ vol/vol). The gels were destained in 7% (vol/vol) acetic acid and 12% (vol/vol) methanol in water as described elsewhere [17]. The molecular weight standards used to construct calibration curves were either intact or cyanogen bromide-generated fragments of horse heart myoglobin (molecular weights = 16,947, 14,404, 8,159, 6,214, and 2,512 daltons). These standards were purchased from BDH Chemicals, Ltd. (Poole, England).

RESULTS

Extraction of MTGF-Pit

Equal amounts of lyophilized powder were extracted under comparable conditions with either phosphate-buffered saline (PBS), pH 7.2, or 0.1 M acetic acid as described in Figure 1. Assays of these soluble supernatants showed that acetic acid extraction yielded 10–14 times more MTGF-Pit activity than was extractable by PBS. Heating the acetic acid extract at 100°C for 5 min did not result in a change in the total or specific activity, although heating the PBS extract at the same temperature caused almost a total loss of activity.

Isolation of MTGF-Pit

A summary of the MTGF-Pit isolation procedure is shown in Table I. From 10 g of dry powder, 8.8 mg of MTGF-Pit was obtained, representing an approximate

TABLE I. Isolation of the Sheep Pituitary Gland Growth Factor

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specificity G_{50} (ng/ml)	Total units of activity	Fold purification[a]	Yield (%)
Acetic acid[b]	86	5.10	439	702	6.2×10^5	1(31.7)	100
Heat treatment	61	2.22	136	176	7.7×10^5	4.0	123
SP-Sephadex C-25	20	2.67	40.1	61	6.6×10^5	11.5	106
Sephadex G-50	20	0.44	8.8	29	3.0×10^5	23.9(758)	48

[a]Fold purification based on phosphate-buffered saline (pH 7.2) extract of lyophilized powder of whole sheep pituitaries is shown in parentheses. The G_{50} of phosphate buffered saline extracts was 22 μg/ml as calculated from data presented in Figure 1.
[b]10 g dry powder extracted with 100 ml of 0.1 M acetic acid.

50% yield. The fold purification based on the specific activity of the acetic acid extract was approximately 24-fold, although if this purification was calculated from the initial activity of neutral phosphate buffer extracts (Fig. 1), the overall increase in activity was 758-fold.

The elution profile of both protein (280 nm absorbing material) and growth factor activity from the final Sephadex G-50 column is shown in Figure 2. It was evident from these data that the major protein peak and the growth factor activity coincided. Calibration of this column suggested a MTGF-Pit elution volume corresponding to an estimated molecular weight of 1,000–10,000 daltons (data not shown). The estimation of molecular weight by Sephadex G-50 eluted in 0.1 M acetic acid was complicated by the fact that the most applicable standards such as insulin and myoglobin (and sequenced fragments of myoglobin) do not elute in volumes corresponding to their expected molecular weights (Stokes radii). Therefore, only a relatively broad estimation of size was possible from Sephadex G-50 elution volume data. A more accurate estimation of molecular weight was made by urea-SDS polyacrylamide gel electrophoresis as shown in Figure 2 (right side). Application of up to 50 μg MTGF-Pit per gel, followed by Coomassie blue staining, revealed one major band (Fig. 2). Estimation of the molecular weight was made as described in Materials and Methods using myoglobin standards of size ranging from 16,949 to 2,512 daltons (Fig. 3). Minor bands were identified in the preparations, as shown in Figure 2, but their apparent molecular weight (migration position) was not consistent from one analysis to another; a densitometer scan of a typical Coomassie blue-stained gel is shown in Figure 4. As shown, the minor bands usually were less than 5% of the total stained material found on the gels.

In parallel experiments, we have attempted to elute MTGF-Pit activity from unstained, frozen urea, SDS gels as described before for the similar 4,200-dalton growth factor purified from pregnant sheep uteri [17]. These experiments yielded no recoverable MTGF-Pit from the eluted gel discs. Thus, confirmation remained to be obtained that the MTGF-Pit activity resides within the major Coomassie blue-stained protein band in the urea, SDS gels. Alternatively, using molecular sieve TSK-200SW high-performance liquid chromatography (HPLC) (Fig. 5), we have shown that all recoverable (>80% of the total MTGF-Pit applied) activity eluted in the single identified peak of A_{280} material. Comparisons of the elution times of MTGF-Pit and the 4,200-dalton growth factor isolated from sheep uteri [17] suggested that these were very similar in molecular weight (data not shown).

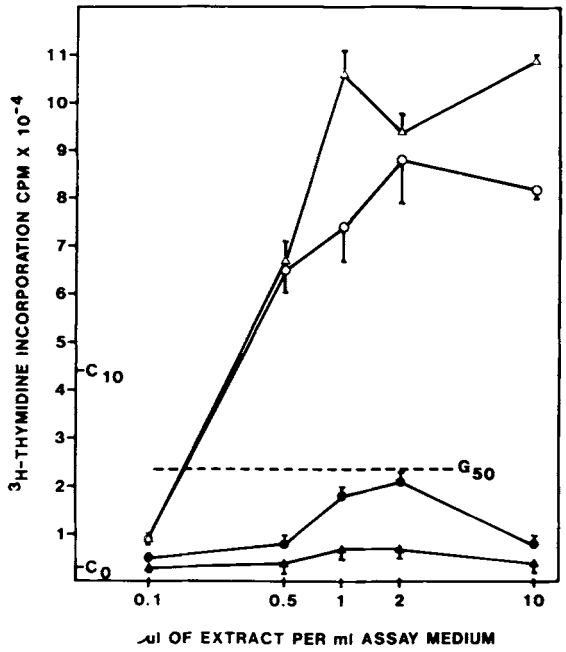

Fig. 1. Extraction of MTGF-Pit activity for MTW9/PL cells from sheep pituitary powders. Portions (5.0 g) of lyophilized sheep pituitary powders were extracted for 24 hr at 4°C with either 45 ml of 0.1 M acetic acid or the same volume of standard pH 7.2 phosphate-buffered saline (PBS). After extraction, the insoluble material was removed by centrifugation at 20,000g. The protein concentrations of the acetic acid extract and the PBS extract were 4.8 and 11.0 mg/ml, respectively. Aliquots (10ml) of each extract were heated at 93°C for 5 min, clarified by centrifugation as above, and compared for MTGF-Pit activity to the corresponding untreated extracts. Designations above are acetic acid extract (open circles), heated acetic acid extract (open triangles), PBS extract (closed circles) and heated PBS extract (closed triangles).

Comparison of the Mitogenic Effects of MTGF-Pit, PRL, GH, EGF, and Fibroblast Growth Factor (FGF) on MTW9/PL Cells

Under serum-free assay conditions the mitogenic effect of MTGF-Pit on MTW9/PL cells was compared to highly purified preparations of bovine-PRL, bovine-GH, mouse-epidermal growth factor (EGF) and bovine pituitary fibroblast growth factor (FGF) (Fig. 6). The G_{50} of purified MTGF-Pit with MTW9/PL cells in serum-free medium ranged between 20 and 30 ng/ml (5.1×10^{-9} to 7.7×10^{-9} M). Neither GH nor PRL were mitogenic for MTW9/PL at concentrations of up to 5 µg/ml. Likewise, EGF and FGF were not mitogenic when tested over concentration ranges corresponding to those used to demonstrate a significant (ie, 10 times serum stimulated) growth in response to MTGF-Pit.

Effect of MTGF-Pit, PRL, and GH on MCF-7 and T-47D Human Mammary Cell Growth

When assayed under serum-free conditions, the human MCF-7 cells responded with significant growth at concentrations of MTGF-Pit between 70 and 200 ng/ml (Fig. 7). The concentrations of MTGF-Pit required to give G_{50} was approximately 75

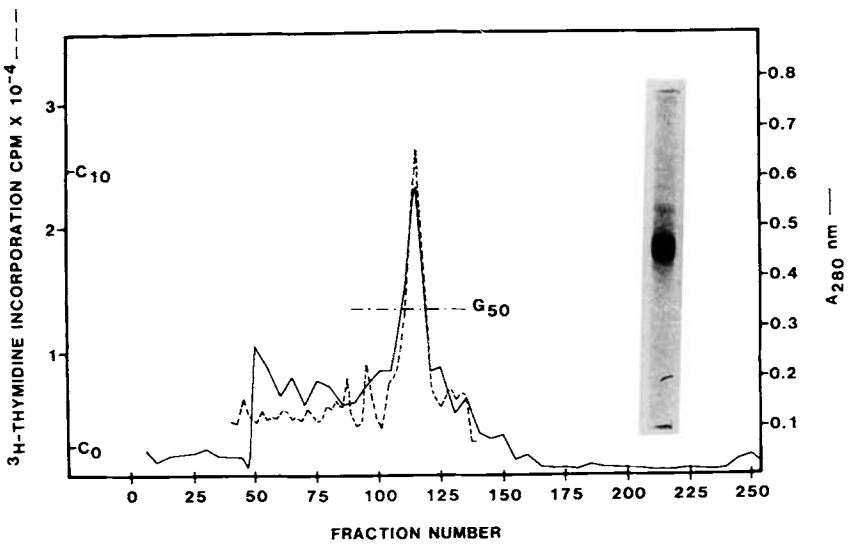

Fig. 2. The elution of PTGF-Pit from the Sephadex G-50 column. The elution profiles of both the 280-nm absorbing material and MTW9/PL cell growth factor activity are shown. The column (3.0 × 115 cm) was equilibrated with and eluted with 0.1 M acetic acid. The individual fractions (4.6 ml each) were assayed for growth factor activity by the procedure described in Materials and Methods. Each fraction was diluted 1,000-fold into the cell growth assays. The most active fractions were pooled and were the final preparation used in this study (MTGF-Pit). The insert on the right side is the 8 M urea-0.1% SDS-polyacrylamide gel electrophoresis analysis of the pooled fraction. MTGF-Pit (50 μg/gel) was analyzed as described in Materials and Methods. The gel electrophoresis was done for 5 hr at room temperature [17], during which time the bromophenol blue marker dye eluted from the gel. The anode is down.

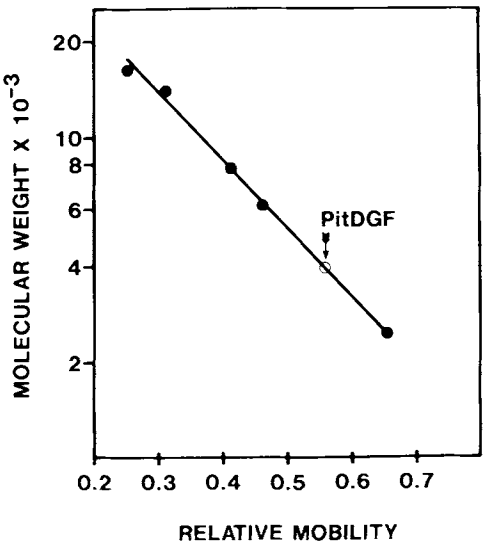

Fig. 3. Estimation of the molecular weight of MTGF-Pit (Pit DGF) by 8 M urea-0.1% SDS-polyacrylamide gel electrophoresis. Gel electrophoresis was conducted for 3 hr at room temperature [17] as described in Materials and Methods. One set of gels received 10 μg of MTGF-Pit while another received 20 μg of a mixture of myoglobin (16,949 daltons) and four sequenced fragments of myoglobin of molecular weights ranging down to 2,512 daltons. The mobilities of MTGF-Pit and the myoglobin standards were expressed as their percent relative mobility compared to migration of bromophenol blue. The molecular weight of MTGF-Pit was estimated as 3,900 ± 200 daltons by this method.

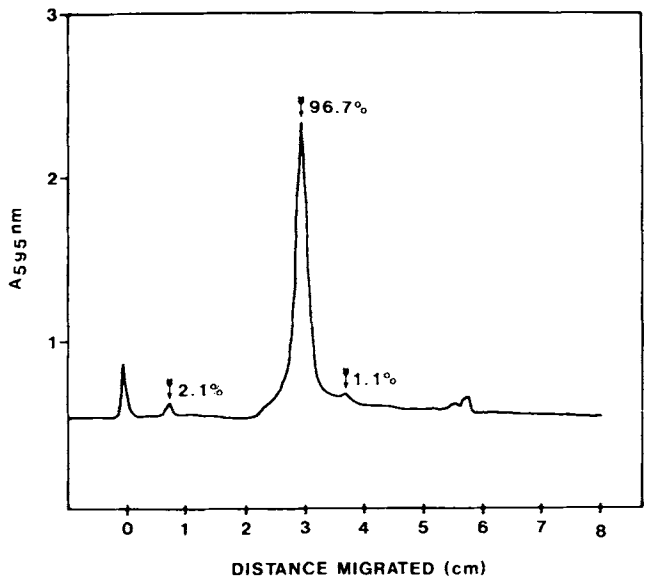

Fig. 4. Densitometer scan estimation of the degree of homogeneity of the MTGF-Pit preparations. A total of 10 μg of MTGF-Pit was submitted to electrophoretic analysis for 3 hr at room temperature [17] as described in the legends of Figures 2 and 3, and after Coomassie blue staining the destained gels were scanned in a Gilford recording spectrophotometer at 595 nm. The percentage that each peak represented of the total absorbing material was determined by integration. Attempts to elute MTGF-Pit activity from parallel unstained urea-SDS gels were unsuccessful, since the growth factor activity was completely abolished by 0.1% SDS treatment. Migration of MTGF-Pit was from left to right.

ng/ml (19.2×10^{-9}M). Under similar conditions, neither bovine PRL nor bovine GH were mitogenic at concentrations of up to 5 μg/ml (approximately 2×10^{-7} M). In the case of MCF-7 cells, the concentration of FCS that promoted optimal tritium-labeled thymidine incorporation into DNA was 5% (vol/vol). Hence, this concentration of serum was used to calculate the G_{50} of MTGF-Pit on MCF-7 cells.

Duplicate experiments (Fig. 8) done with the hormone-responsive human T-47D cells showed a MTGF-Pit G_{50} of 45 ng/ml (11.5×10^{-9}M), while under parallel con ditions, GH and PRL were approximately 100 times less mitogenic (G_{50} = 3 and 5 μg/ml, respectively). In the case of T-47D cells, the serum-promoted incorporation of labeled precursor into DNA was optimal at 2% (vol/vol), hence the G_{50} calculation presented in Figure 8 was based on incorporation promoted by this concentration of FCS.

Effect of MTGF-Pit on GH3/C14 Rat Pituitary Tumor Cell Growth

The effect of MTGF-Pit on GH3/C14 rat pituitary cell growth was assayed by the two different methods outlined in Materials and Methods. The outcome of these studies was that when labeled precursor incorporation was measured within 24 hr of growth factor addition in completely serum-free medium, MTGF-Pit proved to be a potent pituitary cell mitogen (G_{50} = 50 ng/ml). These data are presented in Figure 9b. When labeled precursor addition was measured 48 hr after MTGF-Pit addition, the apparent G_{50} was estimated at 500 ng/ml (Figure 9a). These data demonstrate that selection of the pulse-labeling period markedly influences the calculated potency of a growth factor.

Effect of MTGF-Pit, EGF, and FGF on Growth of Human Fibroblasts

Human fibroblasts maintained in serum-free culture respond to low (ie, 4 ng/ml) concentrations of EGF and FGF, whereas, at concentrations of up to 100 ng/ml, MTGF-Pit had no mitogenic effect on these cells (Fig. 10). It should be noted that under serum-free assay conditions used in Figure 10, neither EGF nor FGF, even at high concentrations, yielded responses that were equivalent to the G_{50} of human fibroblasts. With EGF or FGF, responses of G_{50} magnitude or greater are reported [27,28] to require the presence of a 0.4 to 1% (vol/vol) serum supplement. When the effect of MTGF-Pit was assayed at very high levels on human fibroblasts in serum-free DME, it was possible to achieve a G_{50} response at a concentration of 1.0 μg/ml, which corresponds to 2.5×10^{-4} M MTGF-Pit (see inset Fig. 10). In data not presented, MTGF-Pit at concentrations of up to 1 μg/ml had no mitogenic effect on normal rat fibroblasts.

Effect of Trypsin and Pronase on MTGF-Pit Activity

MTGF-Pit (50 μg/ml) in phosphate-buffered saline (pH 7.2) was incubated with either 1.0 mg of immobilized Trypsin (polyacrylamide covalently attached, 235 units/g dried gel [Sigma Chemical Co., St. Louis, MO]) or 1.0 mg of immobilized pronase (carboxymethyl cellulose covalently attached, 155 units/g of powder, Sigma Chemical Co.). After 5 hr > 99% of the MTGF-Pit activity was lost with the pronase treatment. Trypsin after 5 hr and up to 24 hr caused loss of a maximum of 76% of MTGF-Pit activity (Fig. 11). The data presented show that even after prolonged trypsin treatment residual MTGF-Pit activity can be identified, which might mean that digestion of MTGF-Pit yielded an active, protease stable, core polypeptide. This possibility is

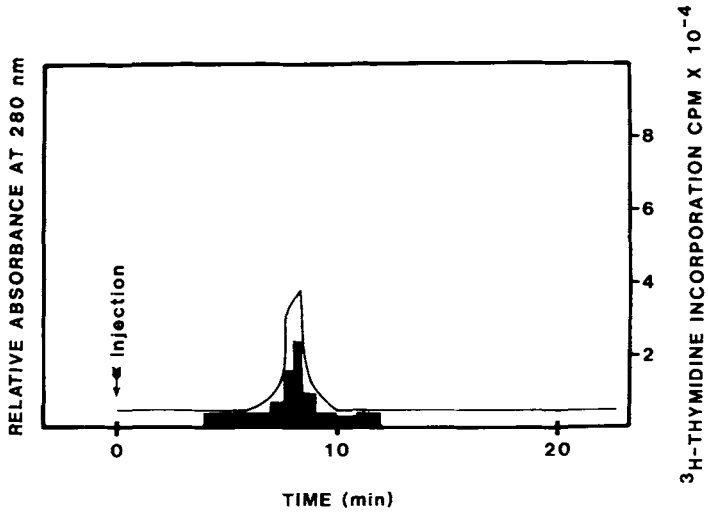

Fig. 5. Molecular sieve HPLC analysis of the MTGF-Pit preparation from the pooled Sephadex G-50 column fractions. A total of 10 μg MTGF-Pit was applied to a (5.2 mm × 30 cm) TSK-2000 SW column equilibrated and eluted with 0.1 M sodium acetate, pH 5.1. The flow rate was 1.0 ml/min. Fractions were collected as shown, each was lyophilized and redissolved in 0.5 ml of distilled water, and 100 μl was used in the standard MTW9/PL cell assay.

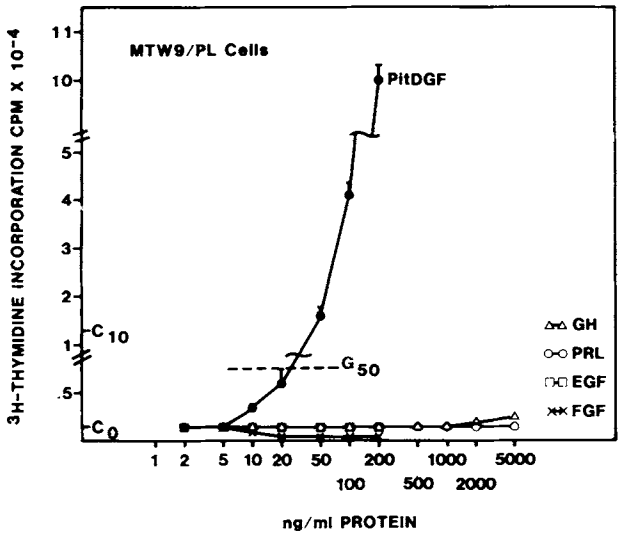

Fig. 6. Effect of MTGF-Pit (PitDGF), bovine GH, bovine PRL, mouse EGF, and bovine pituitary FGF on MTW9/PL cell growth. Assays were conducted as described in Materials and Methods. Purified EGF and FGF were obtained from Collaborative Research Corp. (Lexington, MA).

being explored further. Also, the data presented in Figure 11 extend those of Figure 6 and show that at concentrations of 200 to 500 ng/ml the growth response of the MTW9/PL cells to purified MTGF-Pit reaches saturation kinetics.

Effect of Reducing Agents on MTGF-Pit Activity

Incubation of MTGF-Pit (at 100 μg/ml in sodium phosphate buffer, pH 7.3) in the presence of either 10% (vol/vol) 2-mercaptoethanol or 10 mM dithiothreitol for 30 min at 25°C caused no loss of MTGF-Pit activity toward the mammary tumor cells. When the reduced MTGF-Pit was analyzed by 8 M urea, 0.1% SDS polyacrylamide gel electrophoresis, the apparent molecular weight of 3,900 daltons remained unchanged (data not presented). These results were in contrast to those of similar experiments with other purified growth factors. Others have reported that platelet-derived growth factor (PDGF) [29], EGF [30], and the α and β forms of transforming growth factor (TGF) [31,32] were all inactivated by reducing agents. In the cases of PDGF and TGF the apparent molecular weights changed to lower values and an increased number of bands were identified, suggesting nonidentical subunits covalently attached by disulfide bonds. Comparison of our data with those of the several growth factors cited showed another feature of MTGF-Pit that distinguishes it from these other activities.

DISCUSSION

By methods outlined in this report, a 3,900-dalton polypeptide growth factor has been isolated in high yield by a four-step procedure from normal pituitary gland. Using dissociating conditions of extraction and chromatography in 0.1 M acetic acid, this activity appears markedly different in molecular weight from the well-character-

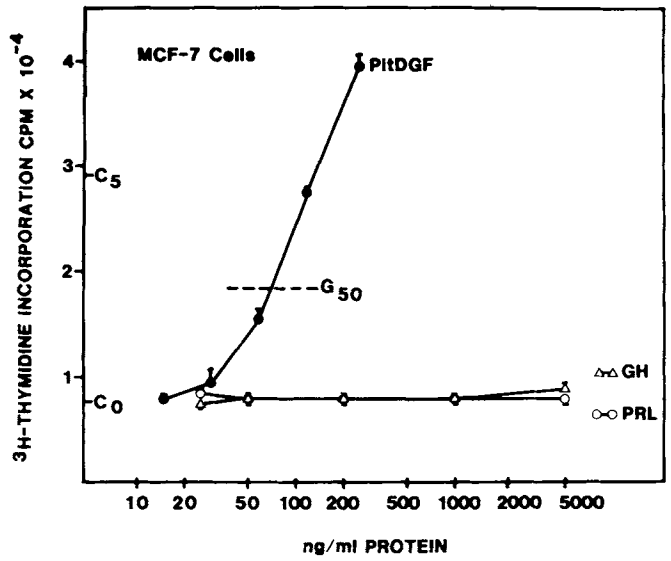

Fig. 7. Effect of MTGF-Pit (PitDGF), bovine GF, and bovine PRL on MCF-7 cell growth. Assays were conducted as described in Materials and Methods.

ized 24,000-dalton form of native PRL or 22,000-dalton form of GH. This apparent large difference in molecular weight between MTGF-Pit, PRL, and GH, as estimated by 8 M urea, 0.1% SDS PAGE, as well as the fact that standard preparations of both PRL and GH do not substitute for this activity, suggest that the MTGF-Pit characterized in this report is new and distinct molecular species.

The observations presented in this report might have considerable biological significance. For several years investigators have been questioning the role of PRL as a mitogenic agent [22,23,33–35]. Clearly, this hormone was directly involved in gene expression leading to milk-specific protein production [36]. However, reports of in vivo studies [22,23], as well as in vitro experiments [33–35], supported the possibility that rather than the well-characterized 24,000-dalton form of PRL's being mitogenic, the pituitary might be the source of either new forms of PRL [22,23] or previously unrecognized growth factors [10,14] that were the active mitogenic agents for mammary gland tissue. Most notably, Mittra [22,23] has reported a 16,000-dalton form of PRL, designated "cleaved PRL," which was a posttranslational modification of the native 24,000-dalton form of the hormone; cleaved PRL showed mitogenic activity in vivo, whereas native 24,000-dalton PRL was inactive [22,23]. Comparison of our data and those of Mittra suggested that MTGF-Pit was not equivalent to cleaved PRL. We emphasize again that this conclusion is supported by the data obtained despite the fact that further characterization of the 3,900-dalton material is required to both confirm the degree of homogeneity and to establish molecular identity by amino acid sequence determination. One possibility not excluded by our data was that MTGF-Pit could be a partial degradation product of the cleaved 16,000 PRL. However, from the reports of Mittra [22,23], we expected that either neutral pH or acidic pH extractions of normal pituitaries [10] or GH3/C14 pituitary cells [10,14] would have yielded identifiable mammary cell mitogenic activity at approximately

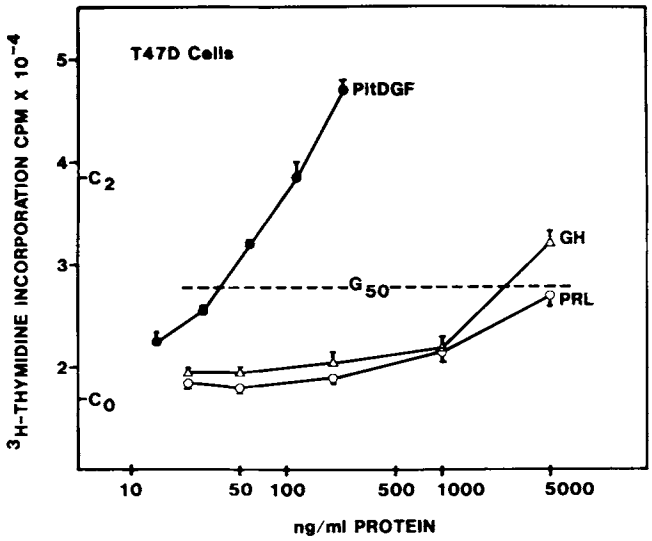

Fig. 8. Effect of MTGF-Pit (PitDGF), bovine GH, and bovine PRL on T-47D cell growth. Assays were conducted as described in Materials and Methods.

16,000 daltons; this has proven not to be the case. As the matter stands, we have not been able to confirm the presence of a 16,000-dalton mitogen (cleaved PRL) in any of our experiments. In any case, the presence of such a considerable amount (0.16% by dry weight) of MTGF-Pit in pituitary powder suggested that sufficient quantities were readily available for performing the necessary amino acid sequence analysis to establish whether MTGF-Pit was a separate new entity, or a specific sequence generated from a known hormone.

In studies now in progress, we are assaying the activity of MTGF-Pit with primary cultures of normal rat mammary gland to determine whether the activity isolated is mitogenic for nonneoplastic mammary. At present the data in this report only support the possibility that this activity is mitogenic for tumor cells. However, since we have already demonstrated that the MTW9/PL cells possess many hormone-responsive properties of normal rat gland cells [6], our data imply a possible function with normal mammary cells.

Further, data presented here (see Fig. 6), as well as in another report [10,17], show that the mitogenic response of mammary cells to MTGF-Pit is not replaced by other known growth factors such as EGF, or the pituitary origin 13,400 molecular weight FGF [28]. Evidence is presented in this report that MTGF-Pit is either not mitogenic for rat fibroblasts, or a very weak mitogen (ie, $G_{50} = 2.5 \times 10^{-4}$ M) for human fibroblasts. This property suggests that MTGF-Pit is not related to mitogens such as EGF or FGF that are both well known for mitogenic activity with fibroblast or mesenchymal origin cells [27,28].

One other aspect of this study also suggests that a potentially important new mitogen has been isolated. We have shown that the GH3/C14 rat pituitary cells respond to low concentrations of MTGF-Pit. If this observation has any physiological relevance, it might be that the pituitary origin growth factor has a role as a locally acting autocrine or paracrine activity for pituitary cells. The only other isolated

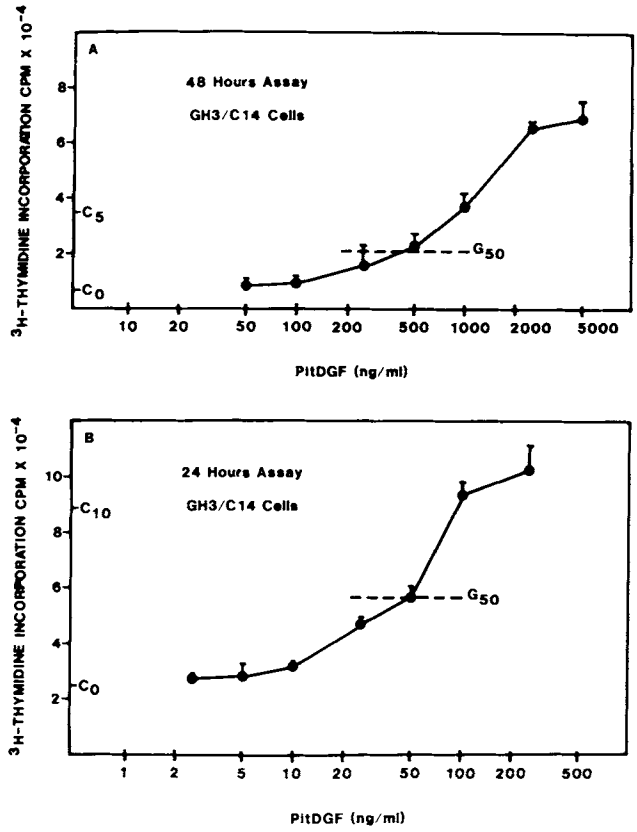

Fig. 9. Effect of MTGF-Pit (PitDGF) on GH3/C14 rat pituitary tumor cell growth in serum-free medium. The growth-promoting potency of MTGF-Pit on GH3/C14 cells was estimated at 48 hr (A) and 24 hr (B) after addition of growth factor to the cells maintained in serum-free medium. The assay procedures are described in Materials and Methods. In the experiment presented in A, the G_{50} of MTGF-Pit was estimated as the concentration of growth factor that half replaced the GH3/C14 cell response to 5% serum, while the data in B expressed G_{50} as half replacement of the response to 10% serum. The reason for the use of two different controls was that these concentrations of serum optimized the cell growth response at these two different labeling times.

growth factor reported to stimulate growth of GH3 cells in serum free culture is IGF-I [37]. We have demonstrated in vivo that serum IGF-I concentrations did not correlate with estrogen-responsive tumor formation by the GH3/C14 clone (T. Ikeda, D. Danielpour, W.H. Daughaday, and D.A. Sirbasku, manuscript submitted), and that IGF-I at concentrations of up to 500 ng/ml was not mitogenic in serum-free culture. Also other growth factors such as the pituitary origin FGF [28] and submaxillary derived EGF [27] were not mitogenic for GH3/C14 cells. Our results with the estrogen-responsive clone of pituitary cells confirm those from another laboratory showing EGF and FGF do not stimulate growth of the parent GH3 cells [38].

Finally, a point must be made about the concentrations of MTGF-Pit required for G_{50} mitogenic responses of the mammary cells in culture. In this report evidence is presented (Figs. 2, 4, 5) that MTGF-Pit is a highly enriched preparation; however, it is recognized that further evaluations of purity must be conducted before conclusive

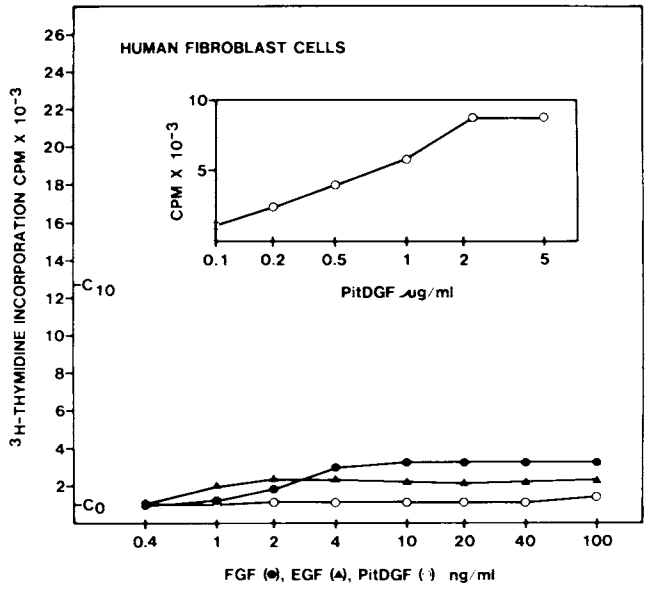

Fig. 10. Effect of MTGF-Pit (PitDGF), bovine pituitary FGF, and mouse EGF on human fibroblast cell growth. Assays were conducted as described in Materials and Methods. Inset shows the effect of high concentrations of MTGF-Pit on human fibroblast cell growth under serum-free conditions.

statements can be made about degree of homogeneity and hence about biological potency. One possibility not excluded by our data is that MTGF-Pit represents only one component of a population of different molecules with nearly identical molecular weights. Hence, further purification by methods such as reverse-phase HPLC might yield a species with even greater biological potency than that currently recognized. Also, with the methods of assay employed in this study, other hormones and growth factors have not been evaluated for synergistic effects with MTGF-Pit. Most notably, the MTGF-Pit activity was assayed under serum-free conditions without the addition of other hormones, metal ion transport proteins, attachment proteins, and growth factors known to be required for optimal mammary cell growth [39]. This method was chosen to ensure the isolation of an independent mitogen. The data obtained showed clearly that the concentrations of MTGF-Pit needed to elicit responses of MTW9/PL, MCF-7, and T-47D cells are 10 to 100 times higher than concentrations described as effective for EGF [27], PDGF [29,40], or pituitary-derived FGF [28] with mesenchymal origin cells. A part of the reason for the differences in biological potency of MTGF-Pit and these other factors might be the fact that the assay methods used to study EGF and to isolate FGF and PDGF utilize serum-supplemented or platelet-poor plasma-supplemented basal medium. This rich basal supplementation is thought to provide the other necessary hormonal and nutrient components that facilitate the action of these factors, decreasing the concentrations required for maximal biological effects. We have presented evidence in Figure 10 that demonstrates EGF and FGF are potent mitogens for human fibroblasts in culture, but if their potencies were estimated under serum-free conditions as the ability to achieve G_{50}, then the effective concentrations of these factors would be $> 10^{-6}$ M. Hence, the biological

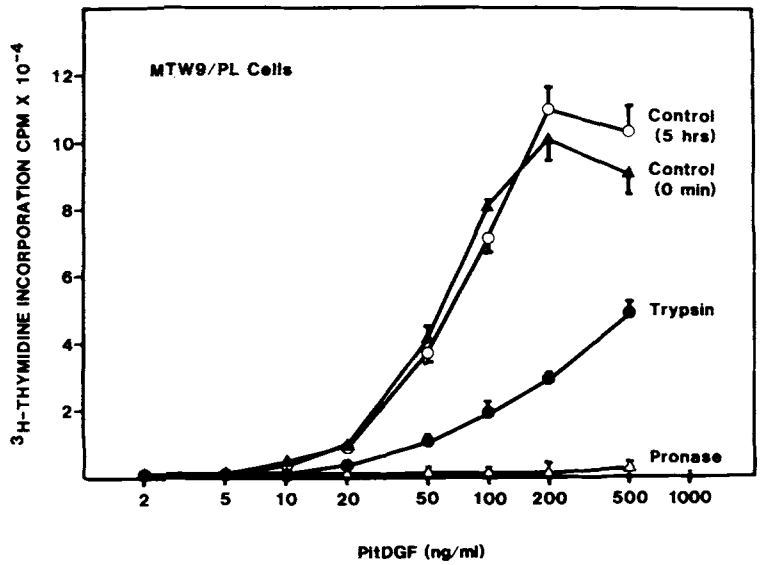

Fig. 11. Effect of trypsin and pronase treatment on the MTGF-Pit (PitDGF) activity toward MTW9/PL cells. MTGF-Pit was incubated with the immobilized proteases as described in the text. After incubation the residual MTGF-Pit activity was estimated by the dose response procedure shown above. The control (5 hr) shown was growth-factor-incubated alone under conditions equivalent to those containing protease (ie, 37°C in buffer). Control (0 min) was MTGF-Pit activity before initiating the experiment.

potency of MTGF-Pit calculated by the methods presented in this report was not directly comparable to those of other factors. For an expanded discussion of this point, see footnote 5 of reference 17. We have attempted to augment the action of MTGF-Pit by serum supplementation but find that even low serum concentrations inhibit MTGF-Pit activity. This finding is consistent with our previous studies [17] of a purified 4,200-dalton mammary tumor cell growth factor isolated from pregnant sheet uterus. The mitogenic activity of this factor was likewise inhibited by serum. From our available data, it is possible that serum contains binding protein(s) for MTGF-Pit and that growth factor in this bound state is inactive. The significance of the binding protein in serum remains to be evaluated.

We are now in the process of further establishing the purity of MTGF-Pit by several methods, and when this work is complete, we will attempt to prepare a serum-free defined medium using MTGF-Pit in mixtures with other known hormones, cell attachment factors, growth factors, and nutrients to replace the serum requirement of MCF-7, T-47D and MTW9/PL cells in culture. At that time, the biological potency of MTGF-Pit will be more accurately assessed.

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