Partial Purification and Characterization of Mammary Stimulating Factor, a Protein Which Promotes Proliferation of Mammary Epithelium†

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ABSTRACT: This report describes the partial purification and characterization of mammary stimulating factor (MSF), a mitogenic peptide isolated from serum which initiates growth in mouse mammary epithelium. By using ion-exchange chromatography, gel filtration, and isoelectric focusing, MSF was purified 250-fold from porcine serum. It is a heat-stable protein of molecular weight 10 100–10 400 with an isoelectric point of 5.5–6.0. MSF initiates DNA synthesis in vitro in mammary epithelium to a greater extent than in mouse mammary tumor cells (CZF), 3T3 cells, or chick embryo cells. Comparison of the biological, physical, and immunological properties of MSF with other established growth-promoting peptides suggests that MSF is a unique serum factor.

Both serum and tissue contain a host of growth-promoting peptides capable of initiating proliferation of most animal cells in vitro (Temin, 1967; Temin et al., 1972). The presence of growth promoting peptides, usually in the form of serum, is required for the growth and maintenance of most tissues in culture. The biological effects of serum on cells in culture include: stimulation of DNA synthesis, cell multiplication, migration, survival, and uptake of low molecular weight nutrients (Lipton et al., 1971; Temin et al., 1972). In an attempt to define those serum components responsible for the initiation of DNA synthesis and mitosis, a variety of growth promoting peptides have been isolated from serum. These include the somatomedins (Hall et al., 1975; Fryklund et al., 1974; Van Wyk et al., 1975), and NSILA-S[1] (Poffenbarger, 1975; Froesch et al., 1967), multiplication stimulating activity (Dulak & Temin, 1973), and platelet factor (Rutherford & Ross, 1976). Factors promoting proliferation that have been isolated from tissue include: EGF (Cohen, 1962) and NGF (Levi-Montalcini & Hamburger, 1953) from submaxillary gland; FGF from pituitary and brain (Gospodarowicz, 1975); MSA from serum-free conditioned medium from buffalo rat liver cells (Nissely & Rechler, 1978); a growth factor isolated from cartilage (Klagsbrun et al., 1977), ovarian growth factor (Gospodarowicz et al., 1974); and MGF from bovine pituitary that is specific for the 64-24 rat mammary tumor cell line (Kano-Sueoka et al., 1977).

Hsueh & Stockdale (1974, 1975) and Stockdale et al. (1966) have shown that both serum and insulin are capable of initiating DNA synthesis in mouse mammary gland epithelium by using organ or monolayer cultures. Serum and insulin appear to act independently or on different cell populations since they produce additive effects even when each is added at its maximally effective concentration. The serum factor(s) responsible for growth in this system (mammary stimulating factor) is precipitated with 50% ammonium sulfate, high in molecular weight at neutral pH, and stable to heat (Hsueh & Stockdale, 1974).

The experiments described here were designed to compare the biological properties of a partially purified preparation of mammary stimulating factor with other growth-promoting peptides capable of initiating DNA synthesis in mammalian cells.

Materials and Methods

Culture Technique. Monolayer cultures of mouse mammary gland epithelial cells were prepared as described by Feldman (1974) with slight modification. Mammary glands from 15–16 day pregnant BALB/c mice were removed aseptically, minced, and dissociated with collagenase (Worthington Biochemical Corp., 5 units/mg of tissue at a concentration of 250 units/mL of medium BHK-21) for 90 min at 37 °C with continuous stirring. Dissociated cells were washed with medium BHK-21 (Grand Island Biological Co.) and centrifuged at 6000*g* for 2 min. The pelleted cells were washed twice more and then passed successively through sterile 308- and 110-μm nylon filters (Nitex Corp.). The cells were suspended in BHK-21 plus 20% fetal calf serum (Grand Island Biological Co.) and 5 μg/mL of insulin and then counted with a Coulter counter or a hemocytometer. The cells were plated at a density of 1×10^5 cells/well in Falcon Multiwell tissue culture plates. After 48 h of incubation, the medium was removed and BHK-21 alone was added. Twenty-four hours later the BHK-21 was removed, and the test medium including 1% BSA was added for an additional 14 h. Other cell cultures utilized and prepared as described above include 3T3 cells, 12-day chick embryo muscle, and CZF (mouse mammary tumor).

Assay for MSF Activity. Serum fractions and growth-promoting peptides were assayed for their ability to stimulate DNA synthesis in mammary epithelium, 3T3 cells, 12-day chick embryo muscle cells, or mouse mammary tumor cells by determining the amount of [^{3}H]methylthymidine (specific activity, 17 Ci/mM) incorporated into acid-insoluble materials. The maximal rate of tritiated thymidine incorporation occurred at 14 h. After 14 h of incubation in the test medium, the cells were exposed for 2 h to medium BHK-21 containing tritiated thymidine (2 μCi/mL). At the end of the tritiated thymidine pulse, the medium was removed and the cells were washed

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[1] Abbreviations used: NSILA-S, nonsuppressible insulin-like activity-soluble; MSA, multiplication stimulating activity; EGF, epidermal growth factor; NGF, nerve growth factor; FGF, fibroblast growth factor; MGF, mammary growth factor; SM, somatomedin; $NaDodSO_4$, sodium dodecyl sulfate; MSF, mammary stimulating factor.

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three times each with 1 mL of cold Hanks' solution and then with cold 5% Cl_3CCOOH. The cells were solubilized with 0.3 mL of 0.5 N sodium hydroxide, and the radioactivity was determined in a Beckman liquid scintillation counter by using acidified Aquasol (New England Nuclear) as counting solution. Previous studies have demonstrated that the incorporation of tritiated thymidine into mammary epithelium is proportional to the number of epithelial cells which initiate DNA synthesis in both organ and monolayer cultures (Hsueh & Stockdale, 1974). For each serum fraction and growth-promoting peptide, a dose response was established by plotting micrograms of protein added against percent stimulation of DNA synthesis above control levels.

Purification of MSF. The flow diagram (Figure 1) shows the sequence of steps in isolation of MSF. Ion-exchange chromatography was performed at room temperature on Dowex AG 50W-X4 columns (100–200 mesh, Bio-Rad Laboratories) measuring 5.0 × 75 cm. The Dowex resin was washed successively with excess volumes of distilled water, 1.2 N NaOH, distilled water, and 0.15 M NaCl. Porcine serum (Grand Island Biological Co.), diluted 1:1 with 0.3 M NaCl, was applied to the column and then eluted stepwise with 0.15 M NaCl, 0.005 M NH_4OH, and 0.1 M NH_4OH. The column eluent was monitored continuously with a Chromatronix spectrometer at a fixed wavelength of 254 nm. The elution profile and the absorption of the individual fractions were qualitatively and quantiatively the same at 254 and 280 nm. The pH of each of the fractions eluted with 0.005 M NH_4OH was adjusted to 6 with 1 N HCl to precipitate a small amount of biologically inactive protein. After removing the precipitate by centrifugation, the pH was adjusted to 7.4 with 1 N NaOH, and the solution was dialyzed against distilled water and lyophilized. Protein content was determined by Lowry's procedure (Lowry et al., 1951).

Gel filtration chromatography was performed with Bio-Gel P-100 (Bio-Rad Laboratories) in 1% acetic acid by using a 3.5 × 50 cm column at a flow rate of 78 mL/h and with Bio-Gel P-10 in 1% acetic acid by using a 2.5 × 50 cm column at a flow rate of 36 mL/h.

Isoelectrofocusing of MSF. An LKB 8101 ampholine column (100 mL, LKB Instruments) was used for isoelectric focusing. In a typical experiment, the dense solution (33% sucrose) contained 1.9 mL of ampholine carrier ampholytes (40% LKB 8141) in a total volume of 55 mL. The active serum fraction from P-10 chromatography was mixed with a light solution (5% sucrose) containing 0.6 mL of the same ampholine carrier ampholytes in 55 mL. Twenty milliliters of the cathode solution (60% sucrose) containing 0.3 mL of monoethanolamine was placed at the bottom of the column. After a linear sucrose gradient was made from the dense and the light solutions, 5 mL of the anode solution (1% sulfuric acid) was layered on top. A linear pH gradient of 3.5–10 was formed by applying 500 V for 48 h to the column maintained at 4 °C. Approximately 50 fractions were collected from the bottom of the column. The pH of each fraction was determined and the isoelectric focused materials were pooled on the basis of the UV absorption (254 mμ), and each fraction was dialyzed extensively against distilled water and then lyophilized. The UV absorption of ampholytes was uniform throughout the pH gradient.

Hormone and Growth Substances. Prolactin and growth hormones were obtained from the Endocrinology Division of the National Institutes of Health. EGF was a gift of Dr. Harvey Herschman and was also prepared in our laboratory by extraction of male mouse submaxillary gland followed by

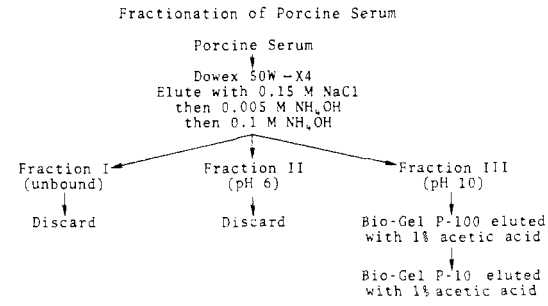

FIGURE 1: Fractionation of porcine serum. Porcine serum was diluted 1:1 with 0.3 M NaCl and eluted stepwise from a Dowex 50W-X4 column by using 0.15 M NaCl followed by 0.005 M NH_4OH. The fraction containing MSF activity (fraction III) was further chromatographed on Bio-Gel P-100 and then Bio-Gel P-10.

column chromatography according to the procedure of Savage & Cohen (1972). FGF was purchased from Collaborative Research, Waltham, MA. Somatomedin (A plus C) was a gift from Dr. Raymond Hintz, and NGF was from Dr. Eric Shooter's laboratory (both of Stanford University). Antibodies of EGF and NGF were gifts from Dr. Harvey Herschmanand Dr. Eric Shooter, respectively.

$NaDodSO_4$ Electrophoresis. The molecular weight of MSF was estimated by using sodium dodecyl sulfate electrophoresis according to the procedure of O'Farrell (1975) with the following changes. Protein (approximately 50 μg) containing bromphenol blue was applied to an acrylamide (15%)–$NaDodSO_4$ (0.1%) slab gel and run at 20 mA constant current for approximately 3 h. The gels were stained in 0.1% Coomassie blue and destained in 50% methanol with 7.5% acetic acid. Standards run included ovalbumin (mol wt 45 × 10^3), chymotrypsin (25 × 10^3), ribonuclease A (13.7 × 10^3), and insulin (6 × 10^3).

Results

Isolation of Growth-Promoting Factor(s). The results of earlier studies (Hsueh & Stockdale, 1973–1975) indicated that serum contains a growth promoting peptide (mammary stimulating factor, MSF) that is a nondialyzable, heat-stable molecule which at neutral pH resides in a fraction that has a molecular weight of approximately 100 000. More recent studies done at a low pH demonstrate that the highest activity resides in a low molecular weight fraction. Further characterization of mammary stimulating factor is described in Figure 1. By using Dowex 50W-X4, three fractions were obtained from whole porcine serum: fraction I washes from the column with 0.15 M NaCl, pH 7.4, and fractions II and III elute at pH 8 and pH 10 (respectively) with 0.005 M NH_4OH.

Fractions I, II, and III were dialyzed, lyophilized, and assayed for their ability to stimulate DNA synthesis in mammary epithelial monolayers as described under Materials and Methods. The percent stimulation of DNA synthesis per mg of protein of fractions I and II is no greater than the whole serum (specific activity, 100), whereas fraction III contains the highest MSF activity (13 000-fold stimulation per mg of protein) and about 0.1% of the total serum protein (Figure 2).

When fraction III from the Dowex column was fractionated by Bio-Gel P-100 (Figure 3), the materials eluting in the molecular size region of ribonuclease A had the highest MSF activity, though there was activity in other regions. The most active P-100 fraction was further chromatographed on Bio-Gel P-10 for a more accurate estimation of molecular weight. The column was calibrated with blue dextran (mol wt 2 × 10^6),

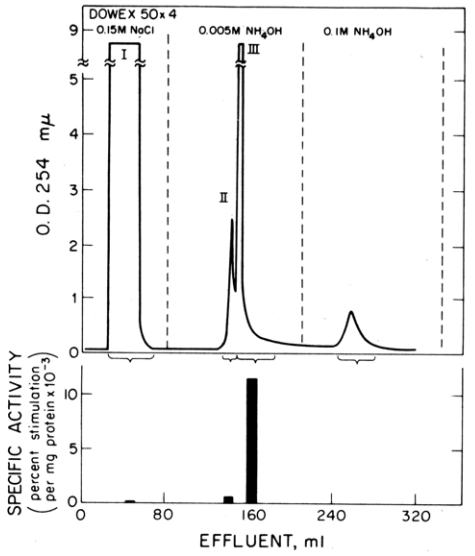

FIGURE 2: Dowex 50W-X4 chromatography. Two liters of porcine serum diluted with 0.3 M NaCl (1:1) was applied to a column (5 × 75 cm) and eluted stepwise with 0.15 M NaCl, 0.005 M NH_4OH, and 0.1 M NH_4OH. The fractions were assayed for MSF activity in mammary epithelium as described under Materials and Methods.

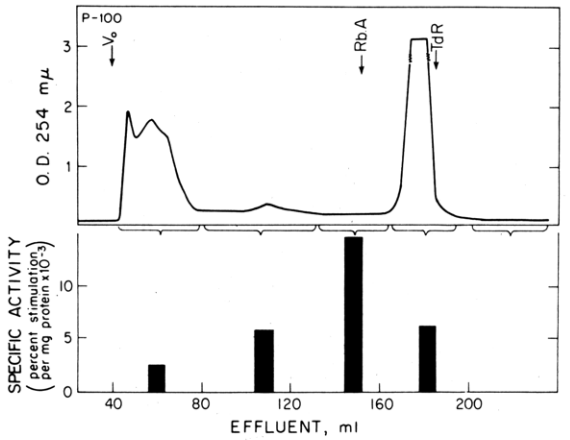

FIGURE 3: Bio-Gel P-100 chromatography. Approximately 100 mg of active Dowex III fraction was applied to a column (3.0 × 50.0 cm) and eluted with 1% acetic acid at a flow rate of 78 mL/h. The fractions were assayed for MSF activity in mammary epithelium as described under Materials and Methods. V_0 = void volume; Rb A = ribonuclease A; TdR = thymidine.

myoglobin (17×10^3), ribonuclease A (13.7×10^3), insulin (6×10^3), and [^{3}H]thymidine (242). Only one region with MSF activity was detected (Figure 4). The molecular weight of material in this region is estimated at 10400 by gel filtration or 10100 by $NaDodSO_4$ slab gel electrophoresis. When the active fraction from the P-10 chromatograph was applied to $NaDodSO_4$ gels, two minor and one major band was found (Figure 5). The major band migrates faster than ribonuclease A, ovalbumin, and chymotrypsin but not as fast as insulin and accounts for 95% of the total P-10 active fraction containing MSF activity. Using a three-step purification (Dowex 50W-X4, Bio-Gel P-100, and Bio-Gel P-10) results in a 50-fold purification of the active factor. The partially purified MSF from P-10 was then applied to an isoelectrofocusing column. A pH gradient of 3.5–10 was established after 48 h at 4 °C with a constant voltage of 500 V. Several fractions were revealed and assayed for their ability to stimulate DNA synthesis. The most active fraction was focused in the region of pH 5.5–6.0 (Figure 6) and was purified approximately 250-fold. The isoelectric point was confirmed by horizontal

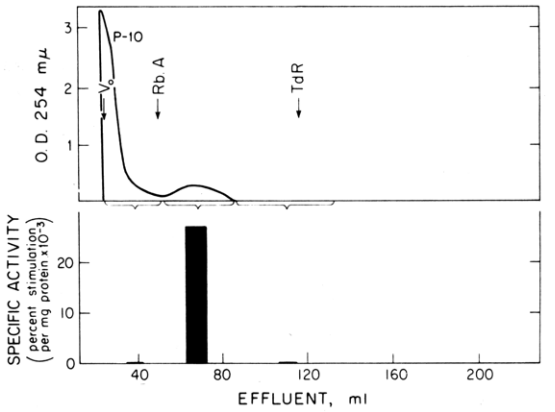

FIGURE 4: Bio-Gel P-10 chromatography. Approximately 3 mg of the most active P-100 fraction was applied to a column (2.5 × 50.0 cm) and eluted with 1% acetic acid at a flow rate of 36 mL/h. The fractions were assayed for their MSF activity in mammary epithelium as described under Materials and Methods.

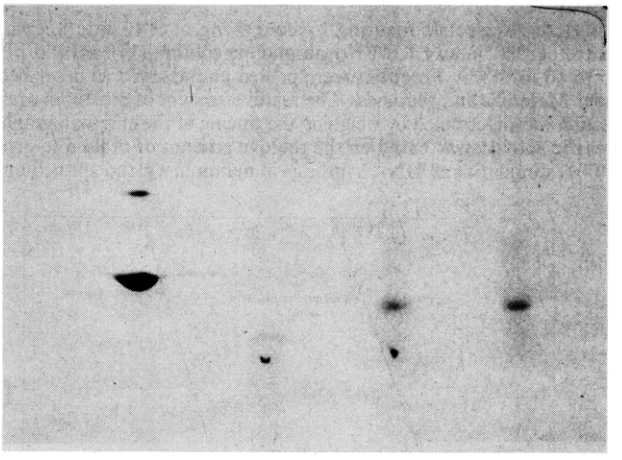

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis. Approximately 50 μg of the protein obtained from the Bio-Gel P-10 column and bromphenol blue was applied to a 15% acrylamide–0.1% $NaDodSO_4$ slab gel of dimensions 14 × 12.5 cm × 1 mm and run for 3 h at 20 mA constant current. The staining procedure is described under Materials and Methods. From left to right the samples were: ribonuclease A (molecular weight 13.7×10^3); insulin (molecular weight 6×10^3) plus MSF; and MSF.

flat-bed isoelectric focusing by using an ampholyte range of pH 4–6. The UV spectra of MSF exhibits a peak at 277 nm, indicating the presence of tryptophan or tyrosine. The presence of tryptophan was confirmed by using molecular circular dichroism.

The biological activity as measured by the rate of DNA synthesis in normal mammary gland epithelium and the degree of purification of Dowex III and P-10 fractions is illustrated in Figure 7 where comparisons are made of these fractions with whole serum. The mammary epithelial cells respond to concentrations of 1–50 μg/mL, but responses to P-10 have been seen with as little as 100 ng/mL. The P-10 fraction is heat stable to 70 °C for 15 min, contains carbohydrates, constitutes 0.0022% of the total serum proteins (~2 mg/L of serum), and loses 20% of its activity by reduction. These studies reveal that the MSF is an acidic protein with a molecular weight of 10100–10400, is heat stable, and may either aggregate or bind to other proteins at neutral pH.

Comparison of MSF with Other Growth-Promoting Peptides. MSF was compared with whole porcine serum, insulin, EGF, NGF, FGF, and somatomedin A plus C in its ability to initiate DNA synthesis in monolayers of mammary epithelium (Figure 8). The effects of various concentrations

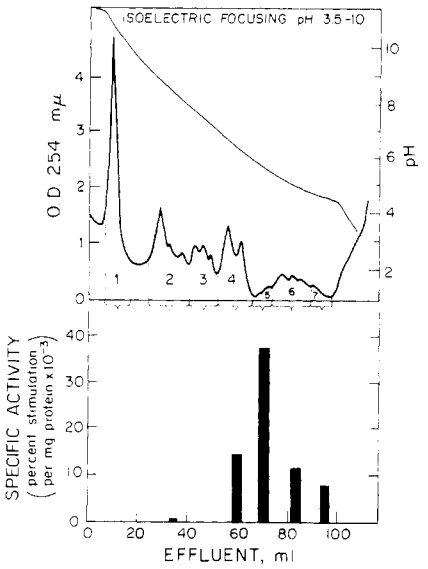

FIGURE 6: Isoelectric focusing. About 6 mg of P-10 fraction was electrofocused on an LKB-8101 ampholine column (110 mL) at pH 3.5 to 10 for 48 h. Fractions were pooled and assayed as described under Materials and Methods. The relative amount of protein in each fraction was determined by weighing the tracing of the chromatograph. Specific activity was based on the relative amount of protein to give a 300% stimulation of DNA synthesis in mammary gland epithelium.

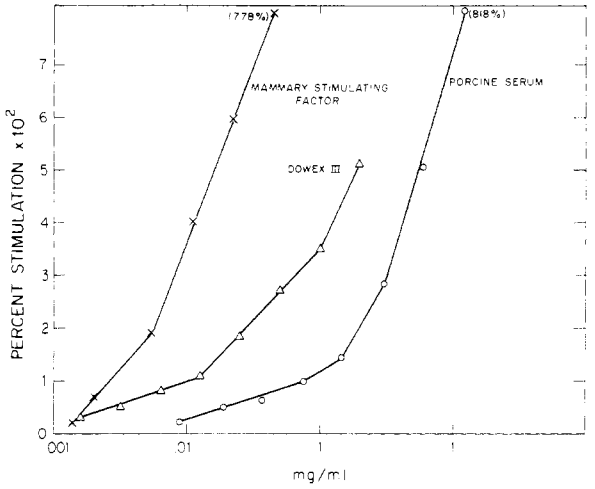

FIGURE 7: Effect of various concentrations of partially purified MSF on DNA synthesis in mammary gland epithelium. Cells were preincubated 48 h with fetal calf serum, insulin, and BHK-21 as described under Materials and Methods. After 24 h in BHK-21 alone, the BHK-21 (control cells) or BHK-21 plus various concentrations of porcine serum, Dowex III fraction, or P-10 fraction was added to the cells. Fourteen hours later, the cells were pulsed for 2 h with 2 μCi of tritiated thymidine/mL and the acid-insoluble counts were determined. Stimulation is reported as percent incorporation relative to cells not exposed to the test substance.

of this growth-promoting substance on titrated thymidine incorporation are shown. DNA synthesis in mammary cells was responsive to porcine serum over a range of 0.1–9.5 mg of protein/mL. MSF (P-10 fraction of Dowex III) stimulates DNA synthesis 380% over control in the range of 0.5–50 μg of protein/mL. EGF stimulates DNA synthesis 350% in the range of 6–500 ng/mL. Cells were responsive to physiological concentrations of insulin (1 ng/mL) with a maximal stimulation of 265% at 900 ng/mL. Porcine serum Dowex III fraction shows maximal stimulation of 250% at 110 μg of protein/mL. Somatomedin (A plus C) was tested between 0.007 and 50 μg of protein/mL resulting in a maximal stimulation of DNA synthesis of 210% at 3.0 μg of protein/mL. Cells were responsive to FGF in the range of 0.01–1 μg of protein/mL with maximal stimulation of 113% at 125 ng/mL. NGF stimulates DNA synthesis 100% at 40.0 μg of protein/mL.

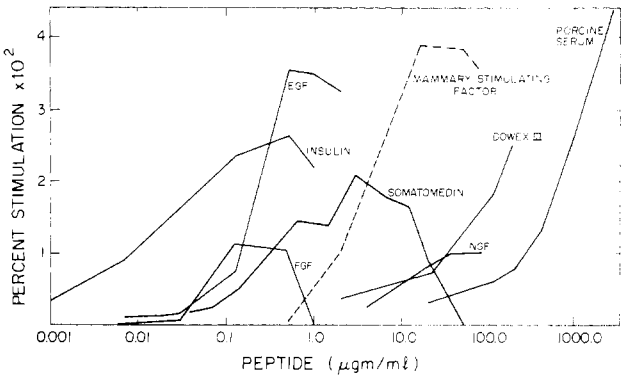

FIGURE 8: Effects of various concentrations of growth factor peptides on the stimulation of DNA synthesis in mammary epithelium. Cells were first preincubated, then incubated with test media and assayed for tritiated thymidine incorporation as described in Figure 7.

The dose–response curves on Figure 8 do not exclude the possibility that MSF and EGF may be the same compound or that small amounts of EGF may be contaminating the MSF. However, the data suggesting that they are separate compounds include: the isoelectric point, MSF (p*I* 5.5); EGF (p*I* 4.6); the molecular weight, MSF (10 100–10 400); EGF (6000); the different elution profiles on Bio-Gel P-10 chromatography; and the lack of cross-reactivity of MSF with antibodies to EGF even at MSF concentrations 50 times higher than that required for EGF to react with this antibody. When MSF and EGF are combined at maximally effective concentrations, they had an additive effect on the number of mammary epithelial cells initiating DNA synthesis. Furthermore, MSF exhibits insulin-like properties since it displaces MSA, somatomedin, or insulin from membrane receptor binding sites, while EGF does not exhibit insulin-like activity (Hollenberg & Cuatrecasas, 1976).

Comparison of MSF with Multiplication Stimulating Activity. The biological activity of MSF was compared with that of multiplication stimulating activity (MSA) in three systems routinely used to evaluate MSA. MSA was purified from serum-free medium conditioned by BRL-3A rat liver cells (Nissley & Rechler, 1978). The assays were performed by Dr. Alan Moses at the Metabolism Branch, NCI, and include the following: a radioimmunoassay for MSA; a rat liver membrane radioreceptor assay for MSA; and a DNA synthesis assay of MSA in tertiary chick embryo fibroblasts.

The radioimmunoassay utilizes an antibody raised in rabbits to a highly purified fraction of MSA. This antibody recognizes only a specific fraction of biologically active MSA, termed the Seph G-75 peak II MSA. MSF and MSA were compared in their ability to displace [^{125}I]MSA from this antibody (Figure 9). The graph shows that Seph G-75 peak II MSA, but not MSF, is able to displace the labeled MSA. Therefore, MSF and MSA appear to be immunologically different. Serum MSA (Pierson & Temin, 1972) also appears to be different from MSF in that on $NaDodSO_4$–polyacrylamide gel electrophoresis serum MSA migrates faster than insulin, whereas MSF migrates more slowly.

The rat liver radioreceptor assay is less specific than the RIA for MSA since the rat liver membrane receptor recognizes not only [^{125}I]MSA but also [^{125}I]NSILA and [^{125}I]SM-A. Displacement of [^{125}I]MSA from rat liver membranes by unlabeled MSA and MSF (Figure 10) indicates that MSF is

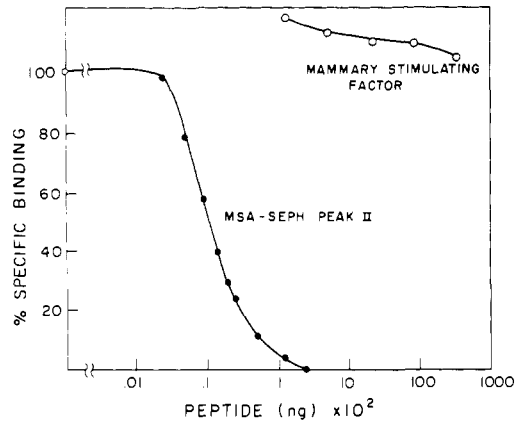

FIGURE 9: Quantitative immunologic comparison of multiplication stimulating activity (MSA) and mammary stimulating factor (MSF). Radioimmunoassay was performed with monospecific antisera raised in rabbits to the biologically active Seph G-75 peak II MSA purified from serum-free media conditioned by BRL-3A rat liver cell line. The graph shows a displacement curve of [^{125}I]MSA from its antibody at 1:1000 final dilution by Seph G-75 peak II MSA.

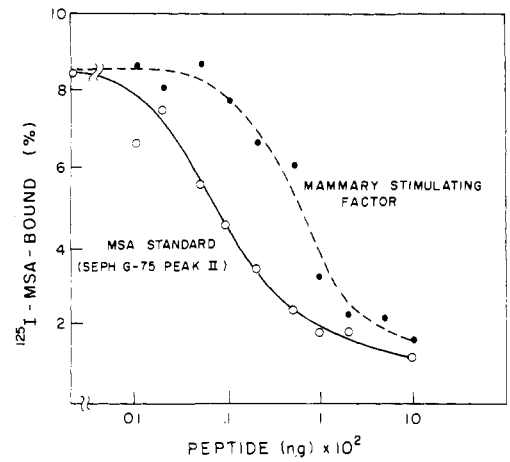

FIGURE 10: Competition for [^{125}I]MSA binding to rat liver membranes by multiplication stimulating activity (MSA) and mammary stimulating factor (MSF). Tracer amounts of [^{125}I]MSA were incubated with an excess of rat liver membrane receptors and increasing concentrations of unlabeled competing MSA and MSF. Half-maximal specific binding of [^{125}I]MSA required 7.5 ng/mL of MSA or 65 ng/mL of MSF.

active in this assay. However, an order of magnitude higher concentration of MSF is required to achieve half-maximal displacement. The results indicate that MSF, like MSA and NSILA, has somatomedin-like activity.

The third assay compares the ability of MSA and MSF to stimulate thymidine incorporation into DNA of chick embryo fibroblasts. MSF stimulates thymidine incorporation at a protein concentration of 6.8 μg/mL with undetectable activity below 1.7 μg/mL, while MSA stimulates thymidine incorporation at 20 ng/mL with detectable levels as low as 7 ng/mL. This assay is not specific since insulin, SM-A, SM-C, and NSILA all stimulate thymidine incorporation under these conditions.

Comparison of MSF with EGF, NGF, and Insulin. Reactivity of MSF with specific antibodies to EGF and NGF was determined. Double immunodiffusion of antibodies and test substance was carried out in agar plates. The center well contained antibodies to EGF and the peripheral wells contained porcine serum, NGF, MSF, EGF, insulin, and FGF. Precipitin bands were seen only against the well containing EGF. The same experiments were carried out with antibodies to NGF and to insulin and the only precipitin bands formed were with

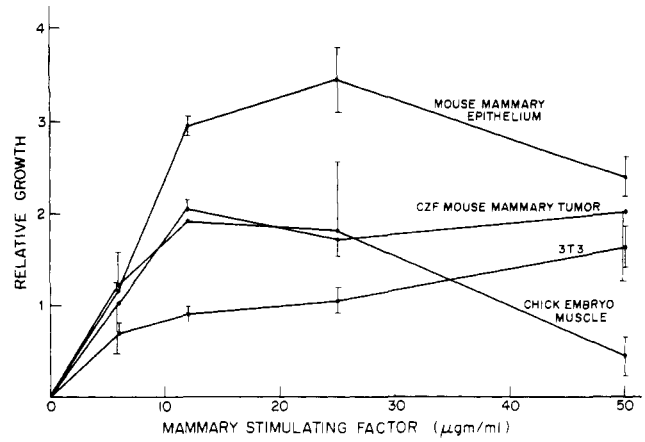

FIGURE 11: Comparison of the effect of mammary stimulating factor on the stimulation of DNA synthesis in various cell types. Cells of mammary epithelium, CZF (mouse mammary tumor), 3T3, or 12-day chick embryo muscle were cultured and incubated with increasing concentrations of MSF as described under Materials and Methods. The relative growth in each cell type in the presence of MSF is expressed as the percentage of stimulation of [^{3}H]thymidine incorporation over that of cells exposed only to medium BHK-21. Each value is the mean of four determinations and the brackets are one standard error of the mean. The control value (BHK-21) was as follows: 3T3, 6324 cpm; tumor, 2153 cpm; mammary epithelium, 8906 cpm; muscle, 705 cpm.

the well containing NGF and insulin, respectively. There was no cross-reactivity with MSF, even at concentrations of MSF 100-fold higher than necessary for the initiation of DNA synthesis in monolayer cultures of mammary gland epithelium. There was no detectable insulin activity in the MSF preparation by radioimmunoassay for insulin.

Comparison of MSF with Somatomedins. Somatomedins are quantitated by a bioassay system in which the uptake of [^{35}S]sulfate by costal cartilage segments from hypophysectomized rats is measured in the presence of test material. This assay performed on MSF by Dr. Raymond Hintz revealed no somatomedin-like activity in the MSF preparation.

By using a competitive displacement assay for ^{125}I-labeled somatomedin, the results showed that MSF displaces somatomedin from a placental membrane preparation at a concentration one order of magnitude higher than that required for somatomedin A plus C to produce displacement.

These observations coupled with differences in isoelectric point (pH 9 for somatomedin vs. pH 5.5 for MSF) suggest that MSF is not somatomedin A or C.

Growth Effects on Cells. In earlier work using autoradiography, we have shown that the addition of serum to mammary cells in organ culture initiates DNA synthesis and increases the percentage of epithelial cells in the population entering DNA synthesis (Hsueh & Stockdale, 1974). MSF also initiates DNA synthesis in normal mammary epithelium, tumor cells from the mammary gland, and several cell types cultured in monolayer (Figure 11). It was important to demonstrate that MSF not only increases the rate of DNA synthesis but also produces a net increase in cell number. Table I shows that MSF fosters cell cycle traverse resulting in a net increase in epithelial cell number in both normal and malignant mammary epithelium. Figure 12 demonstrates the time course of this growth response.

Discussion

Mammary stimulating factor is a small molecular weight acidic protein which initiates DNA synthesis and cell cycle traverse in mammary epithelium. Like most growth factors, it exhibits cross-reactivity at high concentrations with

Table I: Effect of Growth Factors on Cell Number in 24-h Cultures of Normal Mammary Epithelium and Mammary Tumor Cells[a]

cell type	growth factor concn	cell no. × 10^4	% increase[b]
normal mammary gland epithelium	MSF (3 μg)	47	24
	MSF (6 μg)	58	53
	MSF (12 μg)	38	0
	MSF (25 μg)	40	5
	BHK	38	
normal mammary gland epithelium	EGF (2.5 μg)	63	40
	EGF (50 μg)	57	27
	BHK	45	
mammary tumor (CZF)	porcine serum (PS) 10%	97	90
	PS (10%) + MSF (5 μg)	72	41
	PS (10%) + MSF (10 μg)	96	88
	PS (10%) + MSF (25 μg)	42	0
	BHK	51	
mammary tumor (CZF)	porcine serum (PS) 1%	7.05	0
	PS (1%) + MSF (5 μg)	11	54
	PS (1%) + MSF (10 μg)	17.3	142
	MEM	7.15	

[a] Normal mammary gland epithelium and mouse mammary tumor cells were cultured as described in Figure 12. The test medium (MSF, EGF, porcine serum, porcine serum plus MSF, BHK, or MEM) was added for 24 h. The cells were harvested and then counted in a hemacytometer. [b] Percent increase in cell number over control at 24 h.

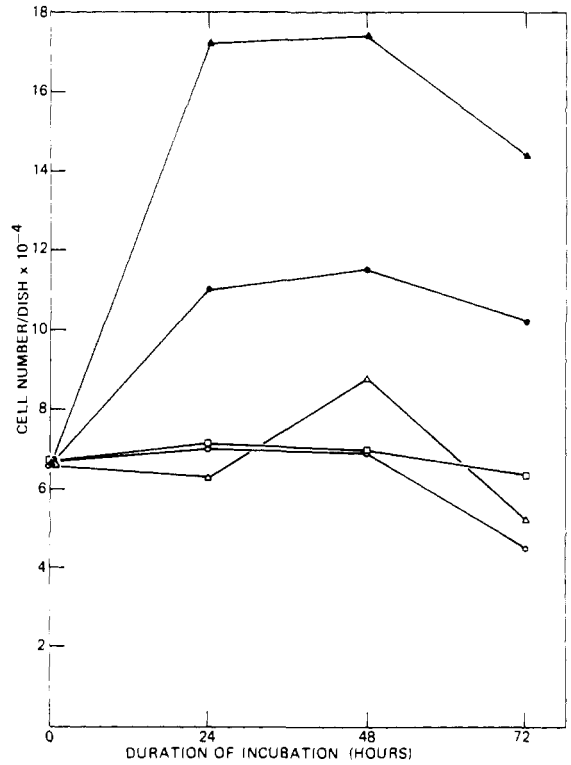

FIGURE 12: Effect of MSF on cell number in 24-, 48-, and 72-h cultures of mouse mammary tumor cells (CZF). Mouse mammary tumor cells were dissociated, washed, filtered, and counted as described under Materials and Methods for epithelial cells, with the exception that tumor cells were further filtered through 35- and 10-μm nylon filters. The tumor cells were plated at a density of 5 × 10^5 cells/35 mm culture dish in medium MEM plus 20% fetal calf serum. After 24 h of preincubation to allow cells to attach to dish, the medium was removed, the dishes were washed, and the cells attached were counted in a hemacytometer (zero time). The test medium, including BSA (5 μg/mL), was changed every 24 h. The test media were: MEM alone (□); 1% porcine serum (○); 1% porcine serum plus 1 μg of MSF (△); 1% porcine serum plus 5 μg of MSF (●); and 1% porcine serum plus 10 μg of MSF (▲). At the indicated time points, the test media were removed from the dishes and the cells were counted in a hemacytometer. Each point is a mean of duplicate determinations.

membrane receptor sites for other growth substances. Therefore, it appears to belong to the class of serum compounds which initiate growth; yet, it differs from insulin, EGF, NGF, FGF, MSA, and somatomedin A and C, and platelet derived growth factor (Antoniades & Scher, 1977; Vogel et al., 1978), by either physiological, biological, or immunological properties.

There are several lines of evidence suggesting that this material is a protein. The ultraviolet absorption spectrum of MSF was observed to be typical for proteins with a 280–260 optical density ratio of 1.4. The isolation procedure for MSF involved standard methods of protein purification. The UV spectra indicate the presence of tryptophan and possibly tyrosine, but not phenylalanine. Molecular circular dichroism spectra of MSF confirmed the presence of tryptophan. MSF reacts with Lowry and Bio-Rad reagents and stains with Coomassie blue as do other growth factor peptides. The isolated material is a partially purified preparation as indicated by several bands on $NaDodSO_4$ electrophoresis.

Of several cell types tested, normal and malignant mammary epitheliums are the most responsive to the growth promoting activities of MSF. MSF appears to initiate DNA synthesis and a single traverse of the cell cycle in these cells. The increase in cell number appears to be limited even though media and growth factors are replenished. The optimal concentration of MSF is in the range of 5–10 μg/mL (0.5–1 μM). Higher concentrations result in less DNA synthesis and cell number increase. Combining optimal concentrations of MSF with low concentrations of serum results in a further increase in epithelial cell growth, while optimal MSF and high serum concentrations do not further increase cell growth.

Neither MSF nor serum will sustain growth of mammary epithelium in vitro beyond a single cell cycle traverse, although mammary epithelium in vitro survives well beyond the time necessary to traverse several more cell cycles. This observation is true with mammary cells cultured in either organ culture or monolayer culture, even in the presence of adequate serum concentrations (10–20%). Other substances in conjunction with MSF may be required for optimal mammary epithelial growth in vivo. On the other hand, there is no evidence to suggest that sustained growth is typical of mammary epithelial cells in vivo. The total growth of the mammary epithelium in the pregnant mouse is equivalent to each cell traversing the cell cycle three times (F. E. Stockdale, unpublished). There is no proliferation of mammary epithelium in the mouse during lactation. Therefore, normal mammary epithelium may have limited potential for sustained growth.

These observations support the contention that there may be a spectrum of growth-promoting serum proteins which initiate growth in a single or limited number of tissue cell types within an organism. Comparisons of MSF with established growth-promoting peptides suggest that MSF is a unique factor. By demonstrating activity in vivo and determining the spectrum of tissue responsiveness, the role of these factors and their relationship with other hormones in growth regulation can be determined.

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Evidence of a Precursor Form of Stratum Corneum Basic Protein in Rat Epidermis†

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ABSTRACT: The fully differentiated anucleate cells of the stratum corneum of newborn rat epidermis contain a cationic protein called stratum corneum basic protein (SCBP). This protein has a molecular weight (49 000) and an amino acid composition similar to a protein extracted from the less differentiated cell layers of the epidermis. Pulse–chase experiments with radiolabeled histidine were undertaken to test the possibility that SCBP is derived from a preexisting protein. A protein of 52 000 daltons is rapidly but transiently labeled in extracts of the less differentiated cell layers. As the amount of label in the 52 000-dalton protein decreases, an increase in radiolabel is observed in extracts of the fully differentiated cells. This label is found in SCBP, a protein of lower molecular weight (49 000) than that initially labeled. These proteins are immunologically related and both are resistant to cyanogen bromide cleavage. They differ in apparent molecular weight on sodium dodecyl sulfate–polyacrylamide gels and in their net charge. The results are consistent with the conversion of a precursor protein into SCBP.

Keratinization is an intracellular differentiation process which culminates in the formation of a layer of fully differentiated anuclear cells, the epidermal stratum corneum. These cells contain the filamentous protein α-keratin and a recently isolated basic protein called stratum corneum basic protein (SCBP)[1] (Dale, 1977). The derivation of SCBP from a precursor is the subject of this report.

Keratinization of normal mammalian epidermis is associated with discrete morphologic changes. Cells at different stages of keratinization are arranged in distinct layers. The basal cells, which lie on the basement membrane separating the

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[1] Abbreviations used: SCBP, stratum corneum basic protein; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; KHG, keratohyalin granule; cpm, counts per minute.

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