Mitogenic Polypeptide of the Mammalian Seminiferous Epithelium: Biochemical Characterization and Partial Purification

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ABSTRACT A mitogenic polypeptide, previously identified in Sertoli cells of the prepuberal mouse (Feig, L. A., A. R. Bellvé, N. Horbach-Erickson, and M. Klagsbrun, 1980, *Proc. Natl. Acad. Sci. USA.*, 77:4774–4778), now has been shown to exist in Sertoli cells of the adult mouse and in the seminiferous epithelium of several other mammalian species, including the rat, guinea pig, and calf. The levels of this seminiferous growth factor (SGF) are not appreciably reduced in adult mouse testes following hypophysectomy. SGF purified from either the adult mouse or newborn calf seminiferous epithelium has a molecular weight (M_r) of 15,700 and a pI between 4.8 and 5.8, when exposed to denaturing conditions. Furthermore, SGF from these two mammalian species probably has few exposed hydrophobic domains and has a strong propensity to aggregate into multiple, high M_r species.

A purification sequence based on these biochemical properties has enabled a greater than 350-fold enrichment of SGF activity from the calf seminiferous epithelium. The protocol involves a sequence of: (*a*) ammonium sulfate precipitation, (*b*) DEAE-cellulose ion exchange chromatography, (*c*) gel filtration chromatography on Bio-Gel P150 in 1.0 M ammonium acetate, (*d*) hydrophobic chromatography on dodecyl agarose, and (*e*) gel filtration chromatography in 6.0 M guanidine hydrochloride. Subsequent analysis of this purified preparation by SDS PAGE, followed by silver staining, reveals approximately 7 polypeptides with M_r between 14,000 and 20,000.

Development of the mammalian testis involves the precise temporal proliferation of somatic and germinal elements. From the initial formation of the primitive gonads near the mesonephros, the expansion and differentiation of the various constituent cell populations appears to be stringently regulated. The precursors of Sertoli cells proliferate rapidly during early fetal stages to form the seminiferous cords of the developing testis (37). In rodent species, the differentiating Sertoli cells continue to proliferate until just after birth, when they become mitotically quiescent (46) while increasing further in size to form the enlarging seminiferous tubules (26). By contrast, Leydig cells first appear later in fetal development, proliferate for a period of time until, just after birth, their numbers become substantially depleted (7, 51). Thereafter, coincident with the onset of spermatogenesis during puberal development, the population of steroid-producing Leydig cells again expands gradually to assume adult numbers. Also, during late fetal development, fibroblasts in the interstitium differentiate to yield the peritubular myoid cells that proliferate continuously to encompass the developing seminiferous epithelium (52). Finally, the migrating primordial germ cells, after settling in the gonadal primordia, continue to divide for a brief time during mid-fetal stages, but then become quiescent for a prolonged period during perinatal development. Shortly after birth, however, the germ cells are stimulated to divide rapidly and differentiate to establish spermatogenesis (for review, see reference 2).

Spermatogenesis in adult mammals involves the mitotic proliferation and renewal of spermatogonia, the growth and meiotic reduction divisions of spermatocytes, and the differentiation of the haploid cells during spermiogenesis (for reviews see references 2 and 5). This sequence of spermatogonia and spermatocyte proliferation is precisely regulated to ensure an orderly, continuous, and abundant production of sper-

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matozoa (for review, see reference 13). Significantly, the expansion of the germ cell population may be regulated by the pituitary gonadotropins and/or by local factors (28). The compensatory testicular growth that follows unilateral gonadectomy of prepuberal animals is associated with elevated levels of serum follicle-stimulating hormone (FSH)[1] (17), but whether this response is due to a direct action of the hormone has yet to be resolved. Other evidence suggests that the division of spermatogonia may be mediated by a testicular "chalone," an inhibitor of cell proliferation (14, 32). But, these latter observations have not been substantiated by others (16). Alternatively, cell proliferation in the testis could be promoted by a mitogenic polypeptide such as the seminiferous growth factor (SGF) that is present in Sertoli cells (23), the somatic cells of the seminiferous epithelium. This concept is based on the known roles of erythropoietin in promoting erythropoiesis (62) and of interleukins 1 and 2 in stimulating the proliferation of lymphocytes (27, 41).

The present study defines the biochemical properties of SGF and also describes a purification protocol that yields highly enriched activity from calf seminiferous cords. These observations represent a significant advance toward elucidating the physiological functions of this novel growth factor.

MATERIALS AND METHODS

Materials

Both normal and hypophysectomized adult CD-1 mice and adult Swiss Webster rats and guinea pigs were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Wild-type, heterozygous, and homozygous mutant mice (W/W^v) were purchased from The Jackson Laboratories (Bar Harbor, ME). Testes from exsanguinated calves were supplied by Trelegans Meat Co. (Cambridge, MA). Microtest II microtiter plates were purchased from Costar (Cambridge, MA), and the 75-μm wire mesh screen was obtained from Newark Wire Cloth Co. (Newark, NJ). Sigma Chemical Co. (St. Louis, MO) provided the hyaluronidase (bovine, type 1-S), trypsin (bovine pancreas, type III), and deoxyribonuclease (DNase I, DN-CL). The Dulbecco's modified Eagle's medium (DME) was supplied by the Grand Island Biological Co. (Grand Island, NY) and the Ham's F12 medium by M. A. Bioproducts (Walkersville, MD). New England Nuclear (Boston, MA) supplied the methyl[^{3}H]thymidine (sp act 6.7 Ci/mM), Schwarz/Mann (Orangeburg, NY) the ultrapure urea and guanidine hydrochloride (G·HCl), and LKB Instruments (Hicksville, NY) the ampholytes (pH 3.5–10).

Assay for Mitogenic Activity

Samples were tested for their ability to stimulate DNA synthesis in confluent, quiescent cultures of BALB/c 3T3 cells (8.0×10^3 cells/0.33 cm^2-microtiter well) that were maintained in 200 μl of DME containing 10% calf serum (22). The incorporation of methyl[^{3}H]thymidine into 3T3 cell DNA was quantified by scintillation spectrometry (33). Background incorporation of [^{3}H]thymidine following addition of PBS was typically <1,500 cpm. Those cells maximally stimulated by addition of fresh calf serum to a final concentration of 20% incorporated ~100,000 cpm. SGF induced DNA synthesis to a maximal level, comparable to or even exceeding that promoted by 20% calf serum. One mitogenic unit was defined as the amount of activity needed to induce half-maximal DNA synthesis in one microtiter well (250 μl) of confluent BALB/c 3T3 cells (8×10^3 cells/0.33 cm^2). The mitogenic polypeptide also promoted cell division in confluent cultures of BALB/c 3T3 cells (23).

Tissue Preparation

Seminiferous cords were prepared from 6-d-old mice and seminiferous tubules were prepared from adult mice and rats. The decapsulated testes were incubated with 0.5 mg collagenase/ml of enriched Krebs-Ringer bicarbonate medium (EKRB) at 33°C, with gentle shaking under 5% CO_2 for 15 min (3, 4, 50). The dispersed seminiferous cords or tubules were allowed to settle and the supernatant fluid was decanted to remove interstitial cells and blood elements. Testes from adult guinea pigs were treated similarly, except that minimal agitation was used during the incubation with collagenase to limit fragmentation of their longer seminiferous tubules.

Seminiferous cords were isolated from 2–3-wk-old calf testes (48) after decapsulating and mincing the tissue into ~1-cm^2 pieces. These segments were placed into EKRB medium containing collagenase (1.0 mg/ml), hyaluronidase (1.7 mg/ml), trypsin (0.5 mg/ml), and DNase (2 μg/ml) and incubated with gentle shaking under 5% CO_2 at 33°C for 20 min. Nondissociated portions of the testes were removed by filtering over a 74-μm wire mesh screen. Segments of seminiferous cords in the filtrate were separated from single cells by centrifugation at 100 *g* for 2 min, and then dispersed briefly in 156 mM NH_4Cl, 100 mM $KHCO_3$, and 130 mM EDTA to lyse contaminating erythrocytes (40). These cord segments were then washed repeatedly by centrifuging and resuspending in EKRB.

Preparations of seminiferous cords or tubules were sonicated (Braunsonic, No. 1510, Braun Instruments, San Francisco, CA) at 100 W for 30 s in either PBS or 1 M ammonium acetate. SGF activity in this preparation could be quantified directly by using the BALB/c 3T3 cell assay. However, usually a cytosolic fraction was prepared by subjecting the sonicated tissue to ultracentrifugation at 100,000 *g* for 1 h. The mitogenic activity was recovered (>90%) in the cytosol. Protein content of all samples was determined by the method of Lowry et al. (35).

Isolation of Sertoli Cells from Adult Mice

Recovery of Sertoli cells was facilitated by using germ cell–depleted animals. In one case, adult mice homozygous for a mutation at the W/W^v gene locus were used. These mutant mice have only Sertoli cells in their seminiferous epithelium; during fetal development their primordial germ cells fail to proliferate and migrate to the indifferent gonads (6, 39). Following collagenase-induced dissociation of the testes from W/W^v mice, separate from those of +/+ and W/+ animals, the respective seminiferous tubule cytosols were prepared and assayed directly for mitogenic activity on BALB/c 3T3 cells. Alternatively, Sertoli cells were isolated from adult mice 30 d posthypophysectomy. A two-step separation procedure was employed in this case, since the seminiferous epithelia of these mice were not totally depleted of spermatogenic cells. After incubating the testes in collagenase, the dispersed seminiferous tubules were recovered and partially dissociated by incubating them in EKRB containing trypsin (0.5 mg/ml) and DNase (20 μg/ml), at 4°C for 15 min in a shaking water bath. The resulting clusters of Sertoli cells and the remaining undifferentiated spermatogenic cells were cultured in a mixture of DME and Ham's F12 (1:1, vol/vol), supplemented with $NaHCO_3$ (1.2 g/l), HEPES (15 mM), glutamine (2 mM), insulin (5 μg/ml), transferrin (5 μg/ml), retinoic acid (50 ng/ml), gentimycin sulfate (50 μg/ml), and fungizone (2.5 μg/ml). This medium supports Sertoli cell growth in vitro (8) and yet does not contain factors capable of stimulating BALB/c 3T3 cell proliferation. Any contaminating germ cells were removed by repeated media changes during days 2–4 of culture. Sertoli cells were identified by their epitheloid morphology and unique, tripartite nucleolus (3, 20). These Sertoli cell preparations, each >90% pure, were scraped from the tissue culture dish and assayed for mitogenic activity on confluent BALB/c 3T3 cells.

Ammonium Sulfate Precipitation

Calf seminiferous cord cytosol was diluted to 6 mg protein/ml of PBS and then solid ammonium sulfate was added to 37% saturation. The sample was stirred continuously for 3 h at 4°C. Precipitated protein lacking growth factor activity was removed by centrifugation at 5,000 *g* for 15 min. Soluble activity in the supernatant was recovered by increasing the ammonium sulfate concentration to 80% saturation, centrifuging the sample at 5,000 *g* for 15 min, and collecting the pellet.

Gel Filtration Chromatography

LOW SALT: Cytosol of mouse or calf seminiferous epithelium was subjected to high performance liquid chromatography (HPLC; Beckman Instruments, Inc., Fullerton, CA) using a Spherogel-TSK G 3000 column (0.75 × 60 cm). Samples of 4 mg protein in 200 μl and the column bed were equilibrated in PBS (pH 7.4) at 20°C. Each sample was applied to the column at a flow rate of 60 ml/h. Aliquots of the 500-μl column fractions were assayed directly for their ability to stimulate DNA synthesis in confluent BALB/c 3T3 cells.

HIGH SALT: Seminiferous cords and tubules from calf and mouse testes, respectively, were suspended in 1 M ammonium acetate (pH 7.2), sonicated, and then subjected to centrifugation at 100,000 *g* for 1 h. Aliquots of the

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; EKRB, enriched Krebs-Ringer bicarbonate medium; FSH, follicle-stimulating hormone; G·HCl, guanidine hydrochloride; HPLC, high performance liquid chromatography; SGF, seminiferous growth factor.

resulting cytosol were chromatographed on a Bio-Gel P150 column (5 × 60 cm), in the presence of 1 M ammonium acetate. Growth factor activity was also chromatographed after being partially purified by ammonium sulfate precipitation and DEAE ion exchange chromatography (see below). This preparative sample, containing 400 mg protein in 40 ml, was applied to a column 10 × 100 cm and eluted at a flow rate of 80 ml/h. All column fractions were lyophilized directly and assayed for growth factor activity.

DENATURING CONDITIONS: Growth factor preparations were also fractionated by HPLC using Spherogel-TSK G3000 SW columns (0.75 × 60 cm, 0.75 × 120 cm) equilibrated in 6 M G·HCl, 5 mM dithiothreitol (DTT). All column fractions were dialyzed and lyophilized before being assayed for growth factor activity.

MOLECULAR WEIGHT DETERMINATION: The M_r of SGF was estimated by plotting $M_r^{0.555}$ vs. K_D, where K_D = elution volume (V_e) − void volume (V_o)/internal volume (V_i) − V_o (25, 47), using data obtained by repeated HPLC of SGF in denaturing conditions. Standard proteins included ovalbumin (M_r = 43,000), chymotrypsinogen (25,000), myoglobin (17,800), ribonuclease (13,700), and insulin α-chain (3,420). V_o and V_i of the columns were quantified from the V_e for blue dextran (M_r = 2 × 10^6) and [^{3}H]leucine (M_r = 131), respectively.

Preparative Isoelectric Focusing

After being partially purified by gel filtration chromatography, the polypeptide's isoelectric point (pI) was determined by using a 110-ml capacity electrofocusing column (LKB Instruments, Bromma, Sweden). Samples containing 5–10 mg protein were dialyzed against 5 mM ammonium bicarbonate and distributed throughout a linear, sucrose gradient (5–50%, wt/vol) containing carrier ampholytes (pH 3.5–10) at 1.8% (vol/vol). In some experiments, 6 M urea and 5 mM DTT were included. The anode electrode solution consisted of 60% sucrose (wt/vol) in 0.15 M H_2PO_4, pH 1.2, while the cathode contained 0.25 M NaOH, pH 11.6. Isoelectric focusing was conducted at 15 W constant power, until 1,200 V was attained. Thereafter, the electrofocusing was continued for 24 h at constant voltage, while the amperage decreased from 8.5 to 1.1 mA. After focusing was completed, 6-ml fractions were collected and their pH was measured at 4°C. Since 6 M urea increases the pI of ampholytes by 0.42 pH U (63), this amount was later subtracted from each measured pH value. The fractions were then dialyzed, lyophilized, and tested for mitogenic activity.

Ion Exchange Chromatography

Growth factor preparations purified by ammonium sulfate precipitation were chromatographed on DEAE cellulose (DE 52; Whatman Laboratory Products Inc., Whatman Paper Div., Clifton, NJ), equilibrated with 10 mM sodium phosphate, pH 6.1. In analytical experiments, samples of 20 mg protein were applied to 1.5 × 10 cm columns and each eluted with a linear 0–200 mM gradient of NaCl. Alternatively, for preparative purposes 2.25 g protein was applied to a 5 × 20 cm DEAE cellulose column and the activity was eluted with a 4-l 0–200 mM NaCl gradient. Flow rates were 30 ml/cm^2-h. Eluted fractions were neutralized by adding 0.5 N NaOH and assayed directly for mitogenic activity on BALB/c 3T3 cells.

Hydrophobic Chromatography

In analytical experiments, SGF activity recovered after gel filtration chromatography was increased in ionic strength by adding ammonium acetate to a final concentration of 4 M. The sample was applied, and after extensive washing with 4 M ammonium acetate, the activity was eluted with a decreasing gradient of 4 M to 0 M ammonium acetate, pH 7.2. Eluted fractions were lyophilized and then assayed for mitogenic activity. In some preparative experiments, SGF was eluted directly with a step gradient of 1.5 M ammonium acetate.

SDS PAGE

Aliquots of SGF activity at all stages of purification were dialyzed against 5 mM ammonium bicarbonate using 6,000–8,000-mol-wt cutoff dialysis tubing and lyophilized. The samples were analyzed by SDS PAGE as described by Laemmli (34), except that 15% polyacrylamide gels were used and the sample buffer contained 2% SDS (wt/vol) and 1.2 M β-mercaptoethanol. Following electrophoresis, the proteins were stained with silver by using the technique of Oakley et al. (45). The silver-stained gels were washed extensively with deionized H_2O before storage. Standards used to estimate the relative M_r of constituents in the SGF preparations included BSA (M_r = 66,300), ovalbumin (43,000), chymotrypsinogen (25,000), myoglobin (17,800), lysozyme (14,300), and cytochrome *c* (12,400).

RESULTS

Mitogenic Activity in Testes of Various Mammalian Species

Seminiferous cords or tubules of mouse, rat, calf, and guinea pig testes were sonicated and tested for their ability to stimulate [^{3}H]thymidine incorporation into DNA of confluent, quiescent, BALB/c 3T3 cells (Table I). The seminiferous epithelium of all species tested contains comparable levels of activity, ranging from 9.7 to 54.0 U/mg protein. Consistent with previous observations (23), testes from prepuberal mice have higher levels of activity than those from adult animals of this species.

Localization of Mitogenic Activity within the Seminiferous Epithelium

Mitogenic activity in the seminiferous epithelium of prepuberal mice is derived primarily from Sertoli cells (23). Whether SGF is localized similarly in testes of adult mice was determined by assaying Sertoli cells that were isolated from animals deficient in germ cells. Germ cell–depleted animals were used because procedures developed for preparing enriched populations of adult rat Sertoli cells (18, 58) do not yield satisfactory results when applied to mice. Two populations of mice were used for this purpose.

Viable, homozygous mutant mice of the genotype W/W^v are characterized by coat color spotting, severe anemia, and infertility due to the absence of germ cells (6). Thus, seminiferous tubules isolated from testes of adult, W/W^v mice by collagenase dissociation yield a >95% pure population of Sertoli cells. Homogenates prepared from these epithelial cells stimulate DNA synthesis in BALB/c 3T3 cells with a specific activity of 44 ± 4 mitogenic U/mg protein (mean ± SE). By contrast, homogenates of seminiferous tubules from heterozygous (W^v/+, W/+) and wild-type (+/+) animals, both containing a normal complement of germ cells, stimulate DNA synthesis at a lower specific activity, 10.7 ± 0.4 mitogenic U/mg protein. The latter level of activity is commensurate with that obtained for testes of adult CD-1 mice (cf. Table I).

Similarly, homogenates of Sertoli cells isolated from hy-

TABLE I

*Growth Factor Activity in the Seminiferous Epithelium of Various Mammalian Species**

Species	Mitogenic activity
	U/mg protein
Prepuberal	
Mouse	44.1 ± 5.2
Calf	54.0 ± 6.5
Adult	
Mouse	11.7 ± 0.6
Rat	9.7 ± 0.9
Guinea pig	20.0

* Seminiferous tubules were isolated from testes of mouse, rat, guinea pig, and calf (see Materials and Methods for details). The tubules were sonicated and the homogenate was assayed for the ability to stimulate DNA synthesis in confluent cultures of BALB/c 3T3 cells. 1 U of mitogenic activity equaled the amount of activity required to stimulate half-maximal DNA synthesis among 8 × 10^3 BALB/c 3T3 cells cultured in a 0.3-cm microtiter well. Data points represent the mean ± SE of repeat determinations on at least four samples, except when indicated otherwise.
Guinea pig data were derived from repeated estimates on a single sample.

pophysectomized mice, which also lack differentiated germ cells, stimulate DNA synthesis in confluent, BALB/c 3T3 cells with a specific activity of 59 ± 9 U/mg protein. This level of activity is comparable to that found for Sertoli cells of adult, W/W^v mutants (44 ± 4 U/mg protein), and again fourfold greater than those of intact, adult seminiferous tubules (see Table I). Since Sertoli cells comprise 24–32% of the volume of the normal adult seminiferous epithelium (12), this data is consistent with these cells being the principal source of mitogenic activity in the mouse testis.

Pituitary Dependence of SGF Activity

Sertoli cells are primary targets for FSH and testosterone (38) and therefore the expression of SGF may be regulated by the pituitary gonadotropins, FSH, and luteinizing hormone. This possibility was assessed by determining the growth factor activity in testes of 30-d-hypophysectomized adult mice. Removal of the pituitary causes a fourfold increase in the specific mitogenic activity of testis homogenates (Table II). But hypophysectomy also decreases total testis protein about sixfold, primarily due to the selective depletion of spermatocytes and spermatids (15), which lack SGF (23). Consequently, the total mitogenic activity per testis decreases only ~35% ($P < 0.002$) (Table II).

Comparison of SGF from Mouse and Calf Seminiferous Epithelia

Growth factor activity elutes as multiple peaks with $M_r >$ 100,000 when calf seminiferous tubule cord is subjected to gel filtration chromatography under nondissociating conditions (PBS; 140 mM NaCl, 1 mM Na_2HPO_4, 2.6 mM KCl, 1.5 mM KH_2PO_4, pH 7.4). By contrast, a single peak of calf SGF, M_r 14,500–17,000, is observed when the cytosolic fraction is chromatographed either in the dissociating conditions of 1 M ammonium acetate (Fig. 1) or in the denaturing conditions of 6 M G·HCl, 5 mM DTT (for example, see Fig. 6). A single ~16,000-M_r activity peak is also observed when any of the multiple, high-M_r forms are rechromatographed in dissociating conditions. Comparable results have been obtained with seminiferous tubule cytosol prepared from adult mice (23). Thus, SGF from both adult mouse and newborn calf has an M_r of 14,500–17,000, but the activity from both sources has a propensity to aggregate into high-M_r complexes.

The pI of mouse and calf SGF was determined by using preparative isoelectric focusing. Growth factor activity from the seminiferous tubule cytosol of both species, after being partially purified by gel filtration chromatography (Fig. 1), is fractionated further by isoelectric focusing in a 3.5–10 pH gradient of ampholytes. A prominent peak of mitogenic activity at pH 3.8–4.2 is observed for both species (Fig. 2). The calf preparation, however, contains a second peak exhibiting a pI between pH 7.8 and 8.2 that represents ~15% of the total activity. Since the growth factor forms high-M_r species in low ionic strength solutions, the pH of fractions in which the two activity peaks are recovered may reflect the pI of some multimer of the protein or a complex formed with another protein(s). Furthermore, many proteins in their native conformation contain ionizable groups displaying abnormal dissociation constants, most likely because these groups form intramolecular bonds and/or are buried within the molecule's tertiary structure (60). This latter possibility can be examined by performing isoelectric focusing in the presence of 6 M urea, which can dissociate oligimers and expose buried ionizable groups (63). In these conditions, the calf preparation still yields two peaks of activity, but now the prominent peak is isoelectric between pH 4.8 and 5.8 (Fig. 2). This basic shift of ~1.0 pH unit, which is also observed for mouse SGF, probably represents the true pI of monomeric SGF. By contrast, the minor activity peak in the calf preparation remains isoelectric at pH 7.8–8.2. Whether this basic growth factor of calf testes is unique to prepuberal animals remains to be determined.

Subsequent efforts were directed toward characterizing and purifying the major acidic growth factor. This protein is partially inactivated at a pH equal to its pI, thereby precluding

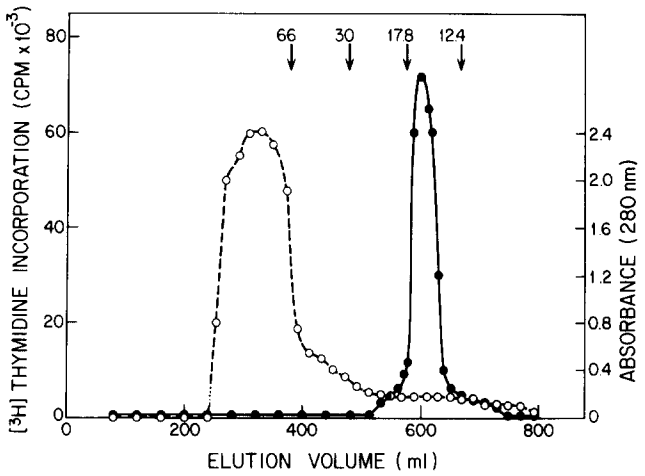

FIGURE 1 Exclusion chromatography of mitogenic activity from calf seminiferous cord cytosol using dissociating conditions. The seminiferous cords were suspended in 1 M ammonium acetate, pH 7.2, sonicated, and subjected to centrifugation at 100,000 *g* for 1 h to remove particulate material. An aliquot of cytosol, 200 mg protein in 10 ml, was applied to a Bio-Gel P150 column (5 × 60 cm) previously equilibrated in 1 M ammonium acetate. The sample was eluted at a flow rate of 30 ml/h and collected in 12-ml fractions that were lyophilized and assayed separately for their ability to stimulate DNA synthesis in confluent, quiescent BALB/c 3T3 cells. The calf mitogenic activity eluted as a single, symmetrical peak with an M_r of 14,500–17,000. Comparable results were obtained when mouse SGF was subjected to chromatography in identical conditions. Standard proteins (× 10^{-3}) include: blue dextran ($M_r = 2 \times 10^6$), albumin (66,300), carbonic anhydrase (30,000), myoglobin (17,800), and cytochrome c (12,400). [^{3}H]Thymidine incorporation (●); absorbance at 280 nm (○).

TABLE II

*Effect of Hypophysectomy on Growth Factor Activity in the Adult Mouse Testis**

Sample	Specific activity	Total protein	Total activity
	U/mg protein	*mg/testis*	*U/testis*
Control	11.8 ± 0.6	11.5 ± 0.9	137 ± 11.3
Hypox‡	47.7 ± 6.1	2.1 ± 0.3	90 ± 4.9

* Testes from five normal, adult mice and seven adult mice 30 d posthypophysectomy were recovered separately, decapsulated, and then sonicated in PBS. The protein content and the specific mitogenic activity (U/mg protein) per testis was determined from each group. From these data the total mitogenic activity in each testis from both normal and hypophysectomized mice was calculated. Data represent mean ± SE.

‡ All values for testes of hypophysectomized mice are significantly different ($P < 0.002$) from the corresponding control values, as determined by two-sample *t* tests.

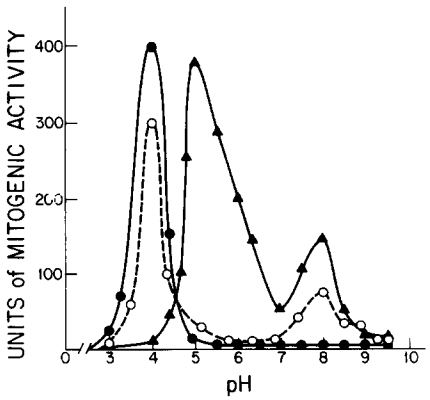

FIGURE 2 Determination of the pI of mouse and calf SGF using both native and denaturing conditions. The respective growth factors were first partially purified from the cytosol of adult mouse and prepuberal calf seminiferous epithelia by gel filtration chromatography (see Fig. 1). These SGF preparations were applied separately to the isoelectric focusing column, either in the presence or absence of 6 M urea, 5 mM DTT (see Materials and Methods for details). After being focused for 24 h, eluant fractions (6 ml) were each measured for pH, dialyzed for 48 h against three changes of 5 mM ammonium bicarbonate, and assayed for their ability to stimulate DNA synthesis. Adult mouse, 10 mg protein (●); prepuberal calf, 4.2 mg protein (○); prepuberal calf, 10 mg protein, in urea (▲).

the use of isoelectric focusing for its preparative purification. Therefore, the calf growth factor recovered by ammonium sulfate precipitation was further fractionated by DEAE chromatography (Fig. 3). While a minor portion (~15%) does not bind to the column, most of the activity elutes between 75 and 175 mM NaCl at a position predictable from the polypeptide's pI (cf. Fig. 2). The broad activity peak probably reflects incomplete dissociation of the monomer in these low salt conditions.

Calf and mouse SGF show a similar degree of apparent hydrophobicity. Both activities, when purified from seminiferous cytosol by gel filtration and then applied to dodecyl agarose in 4 M ammonium acetate (pH 7.2), elute between 3 and 1.5 M on applying a diminishing salt gradient (Fig. 4). In these conditions, >90% of total protein applied remains bound to the column.

Partial Purification of SGF

Calf testes were used as a source for purifying SGF in preparative quantities because: (*a*) the specific mitogenic activity of calf seminiferous cord homogenates is greater than that of seminiferous tubules of those adult species tested (Table 1); (*b*) calf testes can be obtained in reasonable quantities; and (*c*) the biochemical properties of calf and mouse SGF appear to be comparable, suggesting that the polypeptide is phylogenetically conserved.

Routinely, seminiferous cords are isolated from 400 calf testes to eliminate interstitial and blood tissues and hence remove extraneous growth factors. SGF is then partially purified using the scheme outlined in Table III. First, nonmitogenic proteins are precipitated from the seminiferous cytosol by adding ammonium sulfate to 37% saturation. SGF is recovered from the supernatant by increasing the salt concentration to 80% and, after 3 h at 4°C, centrifuging the sample at 5,000 *g* for 15 min. The pellet, containing 2.25 g protein is solubilized in 200 ml of 10 mM sodium phosphate, pH 6.1, and dialyzed against this same buffer. SGF is purified further by DEAE cellulose chromatography (5 × 20 cm) to yield active fractions that, on pooling, show an apparent 10-fold increase in specific activity when compared with the original homogenate (Table III).

The 400 mg protein sample is layered onto a Bio-Gel P150 column (10 × 100 cm), after being concentrated by ultrafiltration and then dialyzed against 1 M ammonium acetate.

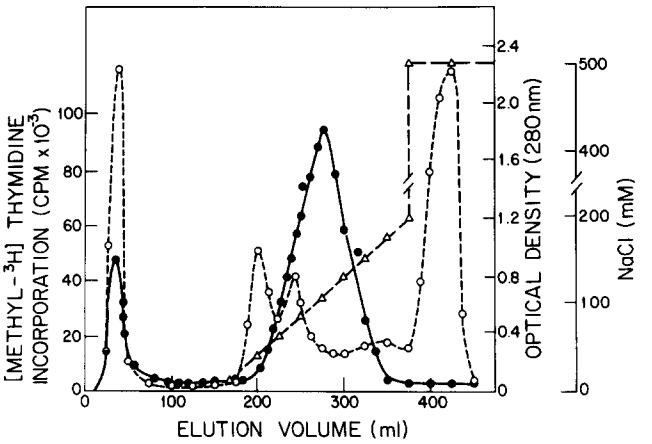

FIGURE 3 Fractionation of calf SGF by DEAE ion exchange chromatography. Growth factor activity of seminiferous cytosol was first partially purified by ammonium sulfate precipitation. The resulting sample, 20 mg protein in 10 mM sodium phosphate, pH 6.2, was applied to a DEAE cellulose column (1.5 × 10 cm), previously equilibrated with the same buffer. Growth factor activity was eluted with a linear, 0–200 mM gradient of NaCl (total volume, 200 ml) at a column flow rate of 60 ml/h. Eluted fractions, 4 ml each, were neutralized by addition of sodium hydroxide and then assayed for mitogenic activity. [³H]Thymidine (●); absorbance at 280 nm (○); NaCl concentration (△).

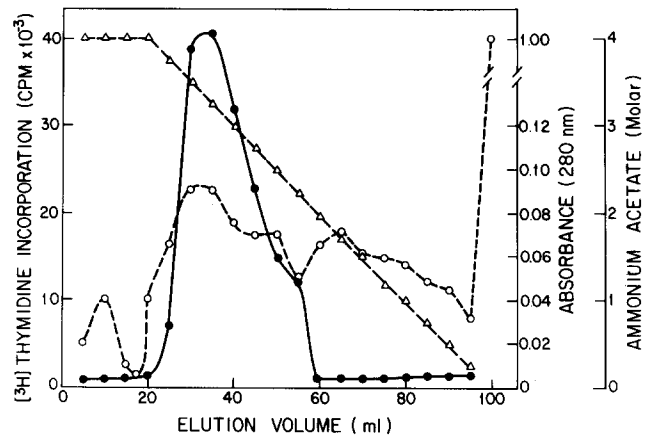

FIGURE 4 Further fractionation of partially purified calf SGF by hydrophobic chromatography. SGF activity was recovered following gel filtration (see Fig. 1), concentrated by ultrafiltration, and then increased in ionic strength by the addition of solid ammonium acetate to a final concentration of 4 M. This sample, 5 mg protein in 10 ml, was applied to a dodecyl agarose column (1.5 × 1.7 cm), previously equilibrated in 4 M ammonium acetate. SGF activity was eluted by a decreasing, linear gradient generated from 35 ml each of 4.0 M and 0 M ammonium acetate, pH 7.2. The 5 ml eluant fractions were lyophilized and assayed for mitogenic activity. Note that >80% of the applied protein remained bound to the column at the completion of the salt gradient. [³H]Thymidine incorporation (●), absorbance at 280 m (○), ammonium acetate concentration (△).

TABLE III

Partial Purification of SGF from Calf Seminiferous Cords

	Protein recovered	Activity recovered*	Overall recovery	Purification factor‡	Half maximal activity§
	mg	*U × 10⁻³*	*%*		*μg/ml*
Tubule homogenate	12,000	300	100	—	160
Cytosol	6,000	270	90	1.8	89
Ammonium sulfate	2,225	200	66.7	3.6	44.0
DEAE ion exchange	400	95	31.7	9.5	16.8
P150 gel filtration	38.0	71	23.7	76.2	2.1
Dodecyl agarose	4.30	35.5	11.8	333.3	0.48
HPLC	0.45	4.0	1.3	355.6	0.45

* One mitogenic unit is the amount of activity inducing half-maximal DNA synthesis in one microtiter well (250 μl) of confluent BALB/c 3T3 cells (8 × 10^3 cells/0.33 cm^2).

‡ The purification factor is the ratio of the specific activity (U/mg protein) of each purified fraction to the specific activity of the original homogenate.

§ Half-maximal activity is the protein concentration at which the pooled, active fractions stimulate half-maximal DNA synthesis in confluent BALB/c 3T3 cells.

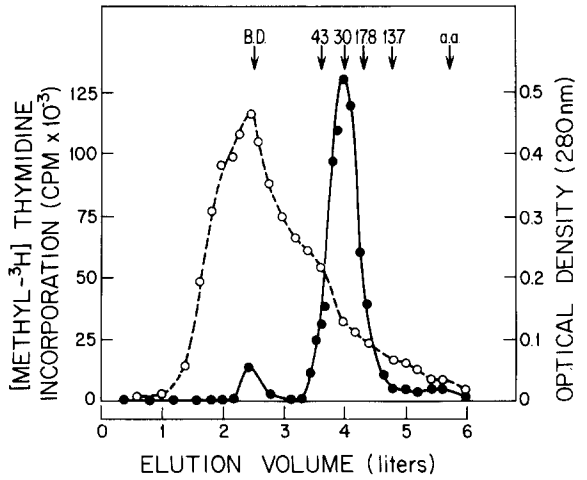

FIGURE 5 Elution of calf SGF as a 30,000-mol-wt species after gel filtration chromatography in 1 M ammonium acetate. Growth factor activity, partially purified by ammonium sulfate precipitation and DEAE ion exchange chromatography, was dialyzed against 1 M ammonium acetate, pH 7.2. The sample, 400 mg protein in 40 ml, was then chromatographed on a Bio-Gel P150 column (10 × 100 cm) equilibrated in the same solution. Aliquots of the eluted fractions (20 ml) were lyophilized and then assayed for mitogenic activity. Calf SGF, when chromatographed under these conditions, eluted as a single peak with an apparent M_r of ~30,000.

Standards (× 10^{-3}) used were blue dextran (*B.D.*, M_r = 2 × 10^6), ovalbumin (43,000), carbonic anhydrase (30,000), myoglobin (17,800), ribonuclease (13,700) and [³H]leucine (a.a., 131). [³H]-Thymidine incorporation (●); absorbance at 280 nm (○).

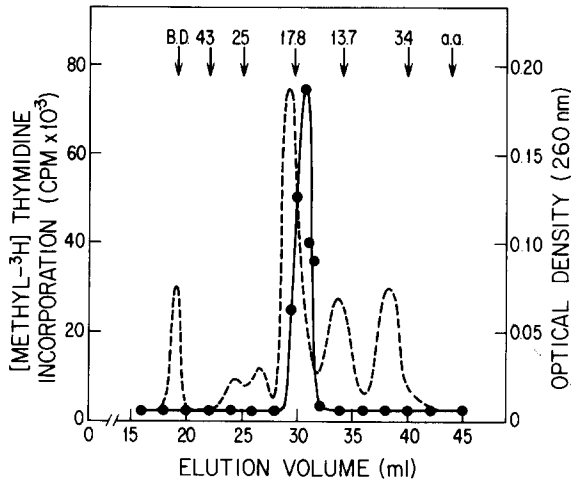

FIGURE 6 Further fractionation of highly purified calf SGF by gel filtration chromatography using denaturing conditions. SGF activity was partially purified from seminiferous cord cytosol by a sequence of ammonium sulfate precipitation, DEAE ion exchange chromatography, gel filtration chromatography, and hydrophobic chromatography. The mitogenic activity was then resolved on two Spherogel-TSK G3000 SW HPLC columns in series (total length, 0.75 × 120 cm). The column and sample, 4 mg protein in 200 μl, were equilibrated in 6 M G·HCl, 5 mM DTT in 20 mM 2,(*N*-morpholino)ethane sulfonic acid, pH 7.0 at 20°C. Elution was carried out at 30 ml/h, and the 2-ml fractions were dialyzed, lyophilized, and assayed for mitogenic activity. The growth factor activity eluted as a single, symmetrical peak with an apparent M_r of 14,500–17,000, and was resolved partially from a major contaminating protein with a slightly larger M_r. Standards (× 10^{-3}) included: blue dextran (*B.D.*, M_r = 2 × 10^6), ovalbumin (43,000), chymotrypsinogen (25,000), myoglobin (17,800), ribonuclease (13,700), α-chain of insulin (3,420), and [³H]leucine (a.a., 131). [³H]Thymidine incorporation (●); absorbance at 260 nm (– – –).

When subjected to these ionic conditions, the growth factor elutes as a single peak corresponding to an M_r of ~30,000 (Fig. 5). Recovery of this presumed dimer of the purified protein contrasts with the 16,000-mol-wt monomer obtained on direct extraction of protein from the crude homogenate (cf. Fig. 1). Thus, even when partially purified, SGF has a propensity to aggregate. Isolation of the growth factor as a 30,000-mol-wt moiety yields an apparent eightfold increase in specific activity at a recovery of 50%.

The purified SGF preparation, now containing 40 mg protein, is subjected to hydrophobic chromatography. In these preparative experiments, growth factor activity applied to the dodecyl agarose in 4 M ammonium acetate is eluted directly with 1.5 M ammonium acetate. This step procedure, rather than the linear gradient (cf. Fig. 4), is used because of its simplicity and comparable resolution of proteins. The specific mitogenic activity is increased another fourfold, and half-maximal DNA synthesis in confluent BALB/c 3T3 cells now occurs at 480 ng protein/ml (Table III).

Finally, after dialysis against 5 mM ammonium bicarbonate and lyophilization, the protein sample (4 mg) is subjected to HPLC (0.75 cm × 120 cm) using dissociating conditions (6 M G·HCl, 5 mM DTT). Repeated analysis by HPLC (25) reveals the precise M_r of SGF to be 15,700 (Fig. 6), as calculated by the procedure of Porath (47) (Fig. 7). During this separation, major contaminating proteins are removed. However, due to a partial denaturation of SGF a considerable

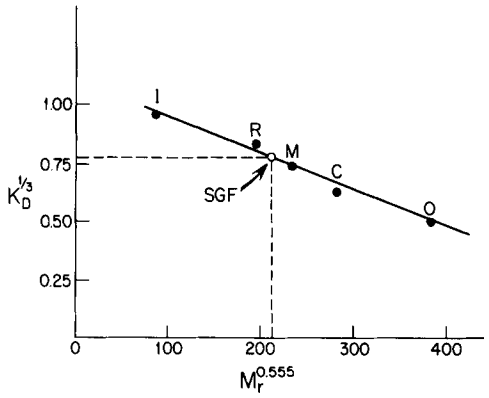

FIGURE 7 Porath plot for M_r estimation of SGF by HPLC in 6 M G·HCl, 5 mM DTT. Values for the distribution coefficient (K_d), where $K_d = V_e - V_o/V_i - V_o$, for SGF and standard proteins of known M_r were derived from the data presented in Fig. 6 and plotted according to the method of Porath (47). An M_r of 15,700 was determined for SGF by plotting the K_d for the polypeptide on a standard curve generated by chromatographing: ovalbumin (O, M_r = 43,000), chymotrypsinogen (C, 25,000), myoglobin (M, 17,800), ribonuclease (R, 13,700), and insulin α-chain (I, 3,420).

amount of the activity is lost (Fig. 7, Table III), and therefore the final preparation again stimulates half-maximal DNA synthesis in BALB/c 3T3 cells at 450 ng/ml (Table III).

The successively purified fractions show marked reductions in protein complexity and a selective enrichment of certain polypeptides when analyzed by SDS PAGE. The final preparation contains approximately 7 polypeptide bands stainable with silver (Fig. 8). Attempts to recover and renature SGF from the polyacrylamide gels have been unsuccessful, and so the identity of the polypeptide band(s) containing mitogenic activity remains to be determined.

DISCUSSION

SGF is a 15,700-mol-wt polypeptide with a pI between 4.8 and 5.8. This mitogen appears to be hydrophilic in its native state, and has a propensity to form high-M_r oligomers or aggregates. Furthermore, SGF activity is resistant to disulfide-bond reduction (23). These biochemical properties distinguish SGF from other well-characterized growth factors, such as epidermal growth factor, platelet-derived growth factor, and the somatomedins, which differ in their M_r and stability properties (11, 53, 64). A growth factor capable of stimulating BALB/c 3T3 cell proliferation has been discovered in rete testis fluid of rams (9); but it, too, appears to be biochemically distinct. However, an endothelial cell growth factor derived from brain may be related to SGF, since it has a similar M_r and pI (36). Significantly, mouse and calf SGF share biochemical properties, and comparable mitogenic activity also exists in the seminiferous epithelium of other mammalian species. Thus, this novel polypeptide appears to be conserved among mammals and therefore may be of fundamental significance to the control of cell proliferation in the testis.

Sertoli cells are the somatic element of the seminiferous epithelium. These epithelial cells, being in intimate morphological association with the germ cells, appear to provide the microenvironment necessary to sustain spermatogenesis. In response to FSH stimulation, Sertoli cells secrete considerable amounts of lactate (49), a metabolic substrate preferred by advanced spermatogenic cells (44), and transferrin (56), an iron-transporting polypeptide required by somatic cells to traverse the G_2 phase of the cell cycle (8, 54). It is significant, therefore, that Sertoli cells of both prepuberal (23) and adult mammalian testes also contain the mitogenic polypeptide, SGF. Unlike these other molecules, however, pituitary hormones do not appear to be major regulators of SGF levels in the testes. Thus, even 30 d after hypophysectomy of adult mice, the total content of growth factor activity in the testis decreases by only ~35%. Although significant, this decrease is modest compared with the 10-fold drop in FSH receptor number (61) and the 1,000-fold decrease in androgen-binding activity (55) in rat testes following hypophysectomy. Furthermore, the level of two other growth factors in mice, epidermal growth factor in the salivary gland (10) and somatomedins in blood (59) decrease 14- and 20-fold, respectively, in response to hypophysectomy.

SGF is presumably involved in regulating cell proliferation in both developing and adult testes. During development, the highest concentrations of mitogenic activity occur in seminiferous cords of prepuberal mice and newborn calves, in a period when Sertoli cells are proliferating rapidly (43). Also,

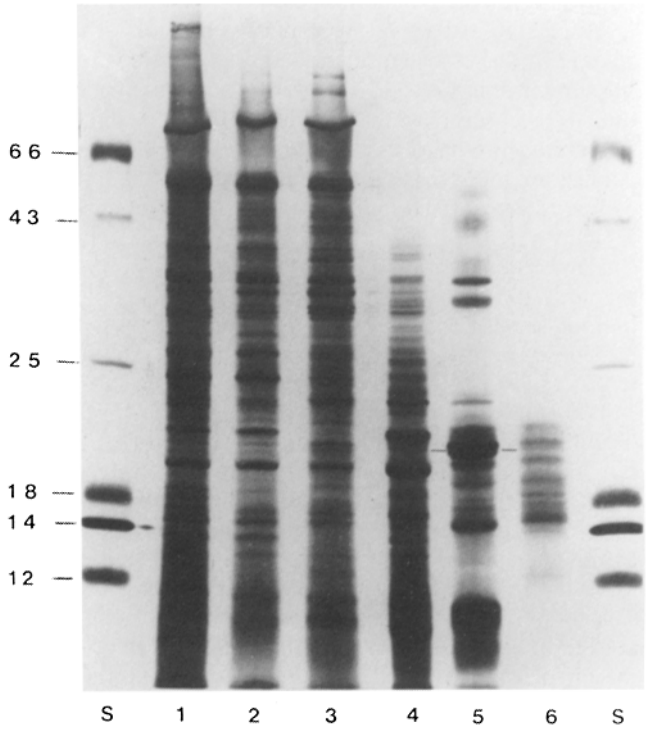

FIGURE 8 SDS PAGE of proteins recovered at successive steps of purifying SGF activity from calf seminiferous cords. Aliquots of SGF activity recovered at each purified step were dialyzed against 5 mM ammonium bicarbonate and lyophilized. These samples were subjected to SDS PAGE (34), and the proteins were stained using the highly sensitive silver technique of Oakley et al. (45). The successive purification steps substantially reduced the complexity of the protein fractions and selectively enriched for certain polypeptides (see mark). After the final purification step (lane 6) only ~7 major polypeptide bands were detectable. The gel lanes contained: (1) crude cytosol; (2) ammonium sulfate precipitate; and the active protein fractions pooled following (3) DEAE cellulose ion exchange chromatography; (4) gel filtration chromatography in 1 M ammonium acetate; (5) dodecyl agarose hydrophobic chromatography; and (6) HPLC in dissociating conditions. Lanes marked (S) contained standard proteins ($\times 10^{-3}$) including: BSA (M_r = 66,300), ovalbumin (43,000), chymotrypsinogen (25,000), myoglobin (17,800), lysozyme (14,300), and cytochrome c (12,400).

the proliferation rate of prepuberal Sertoli cells in vitro is enhanced considerably by SGF (24). By contrast, the differentiated, adult Sertoli cell never divides (57), suggesting that intracellular SGF also may be targeted for other cells in the testis. This potential paracrine function of SGF would be consistent with other evidence suggesting a role for locally derived factors in controlling cell proliferation in the adult testis. For example, depletion of advanced germ cells in the testis by x-irradiation triggers an enhanced mitotic rate among the undifferentiated stem cells, presumably to ensure repopulation of the testis (19, 29). Similarly, Huckins and Cunningham (30) claim that pituitary ablation induces spermatogenic stem cells to increase their proliferative rate. The former response at least could be mediated by local mechanisms, since circulating gonadotropins and intratesticular testosterone levels are not changed (31). Interestingly, the in vivo administration of rat testicular extracts appears to promote replenishment of type A spermatogonia in adult testes previously depleted of the differentiated cells by busulfan treatment (28). This presumptive mitogenic activity is detectable in prepuberal rat testes, but not in similar extracts prepared from adult testes. This observation agrees with the known high levels of SGF activity in the seminiferous cords of newborn mice (23). The biochemical properties of the growth-promoting substance in rat testis have yet to be reported and, therefore, its relationship with SGF is unknown at present. Finally, recent evidence suggests that factors from the seminiferous epithelium influence the growth of cells in the interstitial compartment of the testis (1) as well as the caput epididymis (21).

Clarification of the precise regulatory role of SGF in the mammalian testis will require purification of the polypeptide to homogeneity. The purification scheme employed does not provide an homogeneous preparation of SGF; the final material contains at least 7 polypeptides ranging in M_r from 14,000 to 20,000. Unfortunately, certain properties of SGF have hindered efforts directed toward its complete purification. First, SGF has a strong tendency to aggregate into multiple, high-M_r complexes, necessitating strong dissociating conditions to obtain the monomeric form on gel filtration chromatography. These procedures invariably lead to large losses in activity. Furthermore, this property may be responsible for SGF eluting as a broad peak under the nondissociating conditions used during DEAE chromatography. Second, the biological activity of SGF is far less stable than other growth factors. Unlike epidermal growth factor (11) and multiplication-stimulating activity (42), SGF is inactivated by low pH (22). Therefore, while isoelectric focusing is valuable for analytical purposes, the pH at which the native polypeptide is isoelectric (pI 3.8–4.2) leads to a low yield of activity. These limitations aside, the possibility of purifying SGF to homogeneity in the future remains promising. By using highly purified SGF as an antigen, efforts now are being made to produce a series of hybridoma lines from which monoclonal antibodies to the mitogen may be selected. These monospecific reagents could be used to purify SGF by immunoaffinity chromatography, and also may provide valuable probes for elucidating the physiological function of this novel testicular growth factor.

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