Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2327–2330, April 1987
Developmental Biology

Lentropin, a protein that controls lens fiber formation, is related functionally and immunologically to the insulin-like growth factors

(differentiation/vitreous humor/eye development)

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Communicated by William H. Daughaday, December 11, 1986 (received for review September 17, 1986)

ABSTRACT **Lentropin, a factor present in the vitreous humor of the eye, stimulates lens fiber differentiation from chicken embryo lens epithelial cells *in vitro*. Lentropin has been partially purified but has not been isolated in sufficient quantity or purity for direct comparison with other growth and differentiation factors. Previous studies have shown that insulin and fetal bovine serum share with lentropin the ability to stimulate lens fiber formation from cultured epithelial cells. In the present study, a number of hormones and growth factors were assayed for lentropin activity. Of those tested, the only substances that had this activity were the insulin-like growth factors (IGFs) somatomedin C (Sm-C/IGF-I) and multiplication-stimulating activity (MSA/IGF-II). Sm-C/IGF-I was approximately 30 times more potent than insulin or MSA/IGF-II in promoting fiber cell formation. A monoclonal antibody to human Sm-C/IGF-I inhibited purified Sm-C/IGF-I, fetal bovine serum, and chicken vitreous humor from stimulating fiber cell differentiation *in vitro*. This antibody has been shown not to crossreact with insulin and did not block insulin-stimulated lens fiber formation. These findings indicate that lentropin is related to the IGFs and that these factors may play important roles in controlling cell differentiation, in addition to their better-known ability to stimulate cell division.**

The lens of the eye is composed of two populations of epithelial cells. The anterior surface of the lens is covered by a monolayer of cuboidal cells, which have several important transport functions, whereas the bulk of the lens is composed of elongated fiber cells, which are primarily responsible for the ability of the lens to transmit and refract light. Fiber cells do not normally divide once they are formed. During lens growth new fibers are continually derived from the mitotically active cells at the margin of the epithelium. Cells from the central region of the chicken embryo lens epithelium, which would not normally become fiber cells, will differentiate into fibers when placed in culture medium supplemented with fetal bovine serum (1, 2), insulin (3), or chicken vitreous humor (4). Fiber cell differentiation is characterized by extensive cellular and biochemical specialization, including cell elongation, specialization for the synthesis and accumulation of lens-specific cytoplasmic proteins (the crystallins), cessation of cell division, alteration of the lipid and protein composition of the plasma membrane, and the eventual degradation of nearly all intracellular, membrane-bound organelles, including the nucleus.

Lentropin, the protein in vitreous humor that stimulates lens fiber differentiation, has been characterized and partially purified (4, 5) but has not been obtained in sufficient quantity or purity for structural comparison with other growth and differentiation factors. For this reason we assayed several growth factors and hormones for the ability to stimulate cell elongation in central epithelia dissected from the lenses of 6-day-old chicken embryos. Of the purified factors tested, only members of the family of insulin-like growth factors demonstrated lentropin activity. This result and our subsequent tests indicate that lentropin shares a functional and immunological relationship with somatomedin C/insulin-like growth factor I (Sm-C/IGF-I).

MATERIALS AND METHODS

Unless indicated, reagents were obtained from Sigma, and sera and tissue culture medium, from GIBCO. Human Sm-C/IGF-I was extracted from human serum, or a commercially available version, prepared by recombinant DNA technology, was purchased from AMGen Biologicals (Thousand Oaks, CA). No difference in potency was observed in the growth factors obtained from these two sources. Chicken insulin was a gift of J. R. Kimmel (University of Kansas). Rat multiplication-stimulating activity (MSA/IGF-II) was a gift of S. Peter Nissley (National Institutes of Health, Bethesda, MD). This material had been identified as MSA-III-2 and was a single component when analyzed by HPLC and acrylamide gel electrophoresis. Bovine eye-derived growth factor was a gift of D. Barritault and Y. Courtois (Institut National de la Santé et de la Recherche Médicale, U.118, Paris). Bovine retina-derived growth factor was a gift of P. D'Amore (Harvard University, Boston). Platelet-derived growth factor and fibroblast growth factor were purchased from Collaborative Research (Waltham, MA). Nerve growth factor was a gift of G. Guroff (National Institutes of Health, Bethesda, MD). Cholera toxin was a gift of R. Holmes (Uniformed Services University of the Health Sciences, Bethesda, MD). α-Thrombin was obtained from Bethesda Research Laboratories. Vitreous humor was prepared from vitreous bodies dissected from 15-day-old chicken embryos and centrifuged for 10 min at $12{,}000 \times g$. Sodium bicarbonate (1.2 mg/ml) was added to the supernatant of this centrifugation (vitreous humor), which then was used directly for the culture of lens epithelia (6).

Culture of Lens Epithelia. Fertile chicken eggs were obtained from Truslow Farms (Chestertown, MD), and incubated at 38°C. Lenses from 6-day-old embryos were removed and the central regions of the lens epithelia were explanted into 35-mm culture dishes as described (6). Epithelia were exposed to growth factors or hormones dissolved in Ham's F-10 medium at a concentration of 1 μg/ml, except for

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Abbreviations: IGF, insulin-like growth factor; Sm-C/IGF-I, somatomedin C (IGF-I); MSA, multiplication-stimulating activity (IGF-II).

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15-hydroxyicosatetranenoic acid, which was tested at 1 μM and 100 nM, and the prostaglandins, which were tested at 125 nM. Prostaglandin F_2 was also tested at 12.5 nM. Nitroprusside was tested at 1 mM. Cell length was determined, after incubation of epithelia for 4–5 hr, by measuring the distance between the upper and lower surfaces of the explants with a micrometer built into the focusing mechanism of an inverted microscope (6). Each substance was tested in at least three experiments. Each experiment measured the response of four to six epithelial explants.

Antibody Treatments. Lens epithelia were exposed to fetal bovine serum (15% vol/vol), bovine insulin (1 μg/ml), human Sm-C/IGF-I (100 ng/ml), or chicken embryo vitreous humor (20% vol/vol), all in Ham's F-10 medium, or to the same mixtures pretreated with a 1:1000 dilution of anti-Sm-C/IGF-I monoclonal antibody sm-1.2 (30 min, 37°C). Vitreous humor (5 ml) was also chromatographed over a 1-ml affinity column consisting of sm-1.2 or an unrelated monoclonal antibody of the same subclass (IgG1), mc-9.0, bound to cyanogen bromide-activated Sepharose CL-4B (Pharmacia).

Preparation of Antibody Affinity Columns. Agarose was activated according to a modification of the method of Kohn and Wilchek (7). Ten milliliters of packed Sephadex CL-4B in 60% acetone at −20°C was mixed with 7.6 ml of 1 M cyanogen bromide in 60% acetone, which had been warmed from −20°C with stirring until the cyanogen bromide just dissolved. The mixture was stirred rapidly and 7.6 ml of 1.5 M triethylamine in 60% acetone at −20°C was added dropwise over approximately 1 min. The gel was successively suction-washed on a Buchner funnel with at least five volumes of ice-cold 60% acetone, room temperature 30% acetone, distilled water, and 0.2 M carbonate buffer (pH 9.0). After the final wash, the moist gel cake was suspended in 10 ml of the carbonate buffer containing the immunoglobulin (≈4 mg/ml) to be coupled and then was mixed at room temperature for 4 hr. After coupling, the gel was suspended in an equal volume of 0.1 M glycine (pH 9.0) overnight to block unreacted cyanate esters and then washed and stored in phosphate-buffered saline (10 mM sodium phosphate/0.15 M NaCl, pH 7.4) containing 0.02% sodium azide. Between 10 and 20 mg of the immunoglobulin were bound to 10 ml of the agarose under these conditions.

Protein Synthesis in Cultured Lens Epithelia. Groups of four 6-day-old lens epithelia were dissected, cultured as described above, and incubated in 0.25 ml of Ham's F-10 medium supplemented with [^{35}S]methionine (200 μCi/ml, >1000 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) for 1 hr. The explants then were washed briefly with ice-cold medium, dissolved in 100 μl of electrophoresis sample buffer, and stored frozen at −20°C. Electrophoresis was performed as described (4). After electrophoresis, the gel was dried and autoradiographed, and the autoradiograph was scanned with a densitometer (Quick Scan Jr., Helena Laboratories, Beaumont, TX).

RESULTS

The present study was begun to determine whether other purified growth factors, in addition to insulin, would stimulate lens fiber differentiation *in vitro*. Numerous factors, hormones, and growth modulators were screened for their ability to promote cell elongation in lens central epithelial explants prepared from 6-day-old chicken embryos (Table 1). The only purified factors that were active in this assay were insulin and the insulin-like growth factors, human Sm-C/IGF-I and rat MSA/IGF-II. Factors that were inactive in these experiments included two highly purified preparations derived from bovine retinas, eye-derived growth factor (8) and retina-derived growth factor (9), as well as nerve growth factor, a polypeptide known to promote the survival and differentiation of embryonic neurons (10). Serum from 15-day-old (the age at which vitreous humor was collected) chicken embryos was also inactive.

Table 1. Ability of hormones and growth factors to stimulate lens cell elongation

Treatment	Elongation
Serum (fetal bovine, adult chicken; 5–15% vol/vol)	+
Insulin (avian, porcine, bovine)	+
Vitreous humor (chicken, adult and embryo; undiluted)	+
Sm-C/IGF-I (human)	+
MSA/IGF-II (rat)	+
Serum (15-day-old chicken embryo; 15% vol/vol)	−
Thyrotropin	−
Corticotropin	−
Somatotropin (growth hormone)	−
Lutropin (luteinizing hormone)	−
Follitropin (follicle-stimulating hormone)	−
Chorionic gonadotropin (human)	−
Eye-derived growth factor (bovine)	−
Retina-derived growth factor (bovine)	−
Epidermal growth factor	−
Platelet-derived growth factor	−
Nerve growth factor	−
Hydrocortisone	−
Cholecystokinin	−
[8-arginine]Vasopressin	−
Transferrin (human)	−
Conalbumin (chicken iron-binding protein)	−
Prostaglandins (E_1, F_1, E_2, F_2)	−
15-Hydroxyicosatetraenoic acid	−
Cholera toxin	−
Thryoxin	−
Fibroblast growth factor	−
Prolactin (ovine)	−
α-Thrombin	−
Endothelial cell growth supplement	−
T-cell growth factor (interleukin 2)	−
Melittin	−
Nitroprusside	−
N^6,O^2-Dibutyryladenosine 3′,5′-(cyclic)monophosphate	−

Central lens epithelia from 6-day-old chicken embryos were exposed to test substances for 4–5 hr at a concentration of 1 μg/ml in Ham's F-10 medium, unless otherwise indicated in *Materials and Methods*. Explants that were scored as elongated (+) were >18 μm long and those that did not elongate (−) were <13 μm long at the end of the incubation. At the concentrations used in these tests, no epithelia of intermediate length were detected.

Dose–response tests (Fig. 1) showed that human Sm-C/IGF-I was ≈30 times more effective than insulin in promoting lens cell elongation. Rat MSA/IGF-II had a potency similar to that of bovine insulin.

Increased synthesis and accumulation of the major lens protein, δ-crystallin, is a characteristic of lens fiber cell differentiation in chicken embryos (2). Insulin and serum, in addition to causing cell elongation in cultured embryonic lens epithelial cells, also increase the rate of synthesis and accumulation and the relative percentage of δ-crystallin (11, 12). Autoradiographs of the polypeptides synthesized by lens epithelial cells labeled immediately after explantation or after culture overnight in vitreous humor or in medium supplemented with human Sm-C/IGF-I (100 ng/ml) are shown in Fig. 2. The position of δ-crystallin in the gel is indicated by the arrow. Previous studies have shown that δ-crystallin accounts for ≈8% of the polypeptides synthesized by freshly isolated lens epithelial cells (13). This was confirmed by densitometric scanning of the autoradiographs, which

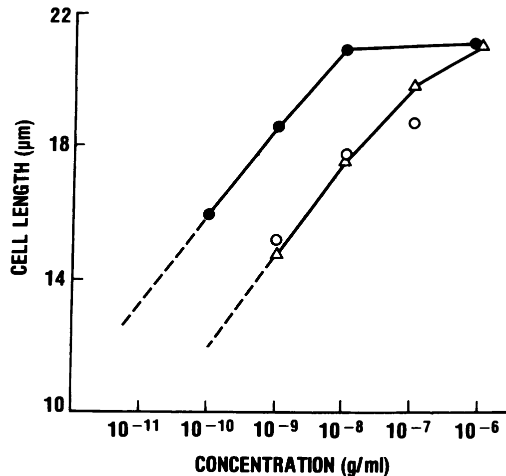

FIG. 1. Cell elongation in response to various concentrations of bovine insulin (△), human Sm-C/IGF-I (●), or rat MSA/IGF-II (○). Cell length was measured as described in *Materials and Methods*. Embryonic lens epithelia cultured in unsupplemented medium did not elongate, and numerous rounded cells accumulated on their apical surface.

showed that 8.1% of the incorporated methionine was associated with the δ-crystallin band in the unincubated epithelial cells in lane 1. Epithelia exposed to either Sm-C/IGF-I (lane 2) or vitreous humor (lane 3) incorporated considerably more radioactivity and a higher percentage of total radioactivity into δ-crystallin (16.5%) than unincubated epithelia (8.1%). Epithelia cultured in vitreous humor incorporated more total radioactivity into protein than those exposed to Sm-C/IGF-I, but the proportion of total synthesis accounted for by δ-crystallin was the same in the two treatments. The increased incorporation in the cells treated with vitreous humor was probably not due to an increase in the number of cells in these explants, because cell division ceases soon after exposure of the cells to vitreous humor or Sm-C/IGF-I (14).

Russell *et al.* (15) have obtained a monoclonal antibody, sm-1.2, that blocks the growth-promoting activity of human Sm-C/IGF-I when tested on cultured mammalian fibroblasts. Preincubation of vitreous humor with this antibody at a dilution of 1:1000 for 30 min substantially decreased lentropin activity (Table 2). Similar experiments showed that sm-1.2 reduced the ability of fetal bovine serum to stimulate fiber cell differentiation and completely blocked the action of Sm-C/IGF-I in this assay (Table 2). Additional inhibition studies with lower concentrations of sm-1.2 showed that it was effective at dilutions of 1:10,000 and, to a more variable extent, 1:100,000 (data not shown).

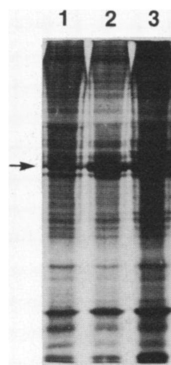

FIG. 2. Acrylamide gel electrophoresis of the polypeptides synthesized by epithelial cells from the central region of 6-day-old lenses. Epithelia were labeled for 1 hr with [^{35}S]methionine immediately after dissection (lane 1), after culture for 24 hr in medium supplemented with human Sm-C/IGF-I at 100 ng/ml (lane 2), or after culture for 24 hr in undiluted chicken embryo vitreous humor (lane 3). The position of the two, unresolved δ-crystallin polypeptides is indicated by the arrow.

Table 2. Inhibition of lentropin activity by sm-1.2, a monoclonal antibody against human Sm-C/IGF-I

Treatment	sm-1.2	Cell length, µm	
Medium alone	−	10.2	($P < 0.2$)
	+	11.0	
Fetal bovine serum (5%, vol/vol)	−	19.6	($P < 0.005$)
	+	14.0	
Chicken vitreous humor (20%, vol/vol)	−	19.4	($P << 0.005$)
	+	14.7	
Human Sm-C/IGF-I (10 ng/ml)	−	19.9	($P << 0.005$)
	+	10.3	
Bovine insulin (1 µg/ml)	−	20.0	($P < 0.2$)
	+	20.7	
Vitreous humor after chromatography			
mc-9.0-Sepharose	−	19.0	($P << 0.005$)
sm-1.2-Sepharose	+	11.2	

Epithelial explants were exposed to fetal bovine serum, bovine insulin, human Sm-C/IGF-I, or chicken embryo vitreous humor, all in Ham's F-10 medium, or the same mixtures pretreated with a 1:1000 dilution of sm-1.2. Vitreous humor (5 ml) was also chromatographed over a 1-ml affinity column, consisting of sm-1.2 or an unrelated monoclonal antibody (mc-9.0) of the same subclass bound to cyanogen bromide-activated Sepharose CL-4B, and then tested directly (100%, no F-10 medium).

sm-1.2 has negligible binding activity for insulin (15). In the current studies the antibody did not prevent insulin from stimulating lens cell elongation (Table 2), and an anti-insulin antibody (generously provided by J. Roth, National Institutes of Health, Bethesda, MD) did not inhibit lentropin activity (data not shown).

An affinity matrix, made by coupling sm-1.2 to crosslinked agarose (7), removed all detectable lentropin activity from chicken embryo vitreous humor (Table 2). Affinity columns prepared from an unrelated monoclonal antibody, mc-9.0, of the same IgG subclass had no effect on lentropin activity.

Sm-C/IGF-I-like immunoreactivity could be detected in chicken embryo vitreous humor by a radioimmunoassay that employed a polyclonal antibody to human Sm-C/IGF-I. However, the binding by immunoreactive material in vitreous humor to this antibody did not parallel that of authentic human Sm-C/IGF-I. Approximately 1 ng of Sm-C/IGF-I-like immunoreactive material was present per ml of vitreous humor (data not shown).

DISCUSSION

The results of these experiments suggest that lentropin is a close structural relative of Sm-C/IGF-I and that the activity in fetal bovine serum that promotes lens fiber cell differentiation is probably bovine Sm-C/IGF-I. The ability of insulin to promote lens fiber formation *in vitro* may be due to its ability to bind to the receptor for Sm-C/IGF-I (16, 17). Both specific (type I) Sm-C/IGF-I receptors and insulin receptors have recently been identified and quantitated on chicken embryo lens epithelial cells (18).

It is likely that monoclonal antibody sm-1.2 and the polyclonal antibody used in the radioimmunoassay do not bind to lentropin with the same affinity that they have for human Sm-C/IGF-I. This may account for the inability of sm-1.2 to block all the lentropin activity in vitreous humor when the antibody was simply mixed with the vitreous humor before being added to the lens epithelia. The observation that the sm-1.2 affinity column removed all lentropin activity from vitreous humor suggests that, if the sm-1.2 concentration is sufficiently high, as it is on the affinity column, all the

lentropin activity can be bound to the antibody. This experiment also suggests that chicken embryo vitreous humor does not contain factors, different from lentropin and Sm-C/IGF-I, that stimulate lens cell elongation.

Little is known about somatomedin/IGF levels during chicken embryo development. In rats, the level of MSA/IGF-II is 20- to 100-fold higher in fetal serum than in maternal serum and declines to adult levels shortly after birth (19). In contrast, Sm-C/IGF-I levels are low in the neonatal rat and rise as the animals mature (20, 21). A similar increase in serum Sm-C/IGF-I is seen during the rapid growth phase in young chickens (22). These temporal changes in serum Sm-C/IGF-I levels may be related to the observation that embryonic chicken serum did not support lens fiber differentiation, although adult chicken serum did (Table 1).

The source of the lentropin found in vitreous humor is not known. The inability of embryonic chicken serum to promote fiber differentiation suggests that lentropin is not transported to the eye in the blood. Several investigators have detected activities in the retina that promote lens differentiation (23), growth (24, 25), or regeneration (26). Experiments performed in our laboratory have detected an activity in the choroid and sclera of the eye of the chicken embryo that promotes cell elongation in cultured lens epithelia (M.H.S. and D.C.B., unpublished work).

A molecule having Sm-C/IGF-I activity is both necessary and sufficient to cause DNA synthesis and mitosis in postmetamorphic frog lens epithelial cells (27). Whether this factor directly stimulates lens fiber differentiation in these animals is not known.

Several recent studies have provided evidence for the importance of IGFs in other examples of cell differentiation. Rat MSA/IGF-II stimulated both the proliferation and the differentiation of rat myoblasts (28, 29). Sm-C/IGF-I promoted the differentiation of ovarian granulosa cells and amplified the stimulatory actions of the classical ovarian effector hormones, estradiol and follicle-stimulating hormone, on these cells (30). A factor in fetal bovine serum that stimulated erythroid differentiation from late erythroid progenitor cells was found to be Sm-C/IGF-I (31). Finally, IGF-I receptors are present at very early stages of chicken embryo development, at which time they are more abundant than insulin receptors (32). These observations show the importance of IGFs in selected examples of cell differentiation and raise the possibility that they play important roles in the early embryo. The present study indicates that lentropin is probably a member of this family of peptides and, in addition, is present in the embryo at the location where lens fiber cells differentiate and at the time when this event is occurring.

Additional information about the amino acid composition and sequence of lentropin will be required before its structural and evolutionary relationship to other IGFs can be determined. This information may be obtained by sequencing the material eluted from Sm-C/IGF-I-antibody affinity columns or by isolation and analysis of chicken embryo cDNA clones for sequence homology with the human Sm-C/IGF-I cDNA, which has been cloned previously (33).

Support was provided by National Institutes of Health Grant EY04853.

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