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Myogenic growth factor present in skeletal muscle is purified by heparin-affinity chromatography

(muscle growth/regeneration/replication stimulation)

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ABSTRACT **A myogenic growth factor has been purified from a skeletal muscle, the anterior latissimus dorsi, of adult chickens. In the range of 1–10 ng, this factor stimulates DNA synthesis as well as protein and muscle-specific myosin accumulation in myogenic cell cultures. Purification is achieved through binding of the factor to heparin. The factor is distinct from transferrin and works synergistically with transferrin in stimulating myogenesis *in vitro*.**

Chicken embryo extract and serum (1), selected skeletal muscles (2, 3), and sciatic nerve (4) extracts all contain transferrin (Tf) or a Tf-like molecule that stimulates myogenesis *in vitro*. We now report (5) that extracts of selected chicken muscles contain a myogenic-stimulating activity in addition to Tf. We also report here a 40,000-fold purification of this material to yield a new factor, chicken muscle growth factor (CMGF). CMGF may be purified from chicken anterior latissimus dorsi (ALD) muscles by binding to a heparin-affinity column followed by elution at high salt concentrations. In nanogram amounts, it stimulates myoblast proliferation and thereby delays temporarily myogenic cell fusion and muscle formation *in vitro*.

The role of muscle growth factors in regeneration and their involvement in activating satellite cells (6) is not known, but physiologically occurring mitogens may be involved. A myogenic cell growth factor has been reported to be released from crushed skeletal muscle (7) and stimulation of smooth muscle cell growth occurs upon injury of the vascular wall (8). Chicken skeletal muscle cells require iron-saturated avian Tf for survival, proliferation, differentiation, and maintenance *in vitro* (1). Other factors known to affect myogenesis in culture include (*i*) multiplication-stimulating activity, insulin-like growth factors I and II (9, 10), which promote differentiation of myotubes, and (*ii*) fibroblast growth factor (FGF), which stimulates myogenic cell division and represses or temporarily delays terminal differentiation (11, 12). FGF was suggested recently to be a potential regulator of satellite cell proliferation in skeletal muscle (13).

Tf has been shown to be selectively accumulated in the interstitial spaces of chicken ALD muscle (3) and was considered to be the factor responsible for the strong myogenic-stimulating effects that ALD extracts exert on muscle cell cultures (2). Moreover, we show below that another growth-promoting activity is present in these extracts. This activity is subject to heat, trypsin, and acid inactivation, is correlated with the presence of a family of basic peptides in the M_r range of 14,000–17,000, and stimulates myoblast division while inhibiting the onset of cell fusion and terminal differentiation. The activity thus shares some of the characteristics of FGF (5, 11).

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MATERIALS AND METHODS

Heparin-Sepharose Chromatography of Muscle Growth Factor. ALD muscles from adult chickens were purchased in lots of 1000 from Pel-Freez. The muscles were kept frozen until use. All isolation procedures were carried out at 0°C unless stated. The muscle was minced and extracted as described by us for Tf isolation (2, 3). After centrifugation of the extract for 1 hr at 100,000 × *g*, the supernatant was dialyzed against 0.2 M NaCl/10 mM Tris·HCl, pH 7.0, and brought to 0.6 M NaCl by using solid NaCl. One gram of this extract, at 8 mg/ml, was absorbed to the heparin-Sepharose column at room temperature. Subsequent elution was carried out at room temperature. The column contained 8 ml of heparin-Sepharose processed according to manufacturer's instructions (Pharmacia). After absorption of the extract, the column was washed extensively with the absorption buffer. Column elution was carried out by using increasing concentrations of NaCl as described in the text. In one round of binding and batch elution, the CMGF elutes at 2 M NaCl. In a second round of chromatography using a gradient elution, CMGF elutes at between 1.4 and 1.5 M NaCl as a family of five peptides of apparent M_rs between 14,000 and 17,000. No protease inhibitors were used during extraction since they interfere with subsequent biological assay.

Growth Factor Assay. CMGF activity was assayed in myogenic cell cultures prepared as described (3). Cultures were grown in complete medium [10% selected horse serum/1.5% embryo extract/minimal essential medium (MEM)] unless otherwise stated. In certain cases, embryo extract was omitted and medium was supplemented with chicken Tf. Heparin column fractions and other additions were made on day 1 of cell culture, usually at the time of cell plating. DNA synthesis was measured by [^{3}H]thymidine incorporation into DNA (31). At the time of plating [^{3}H]thymidine was added (5 μCi/ml; 1 Ci = 37 GBq) together with muscle extract or purified CMGF, and thymidine incorporation into DNA was measured 17 hr later. Myosin heavy chain (MHC) and total protein measurements were carried out as described (14, 15). Briefly, total protein was determined by using a Bradford assay with serum albumin as a standard. MHC was determined by scanning NaDodSO$_4$/PAGE gels of prestained myosin extracts of cell cultures. The MHC band was identified by comigration with purified MHC marker (15).

Electrophoresis of Heparin Column Fractions. Fractions from heparin-Sepharose columns containing CMGF activity were analyzed by standard NaDodSO$_4$/PAGE (16) using 12.5% acrylamide gels. Gels were stained with Coomassie brilliant blue after overnight fixation in 50% methanol/10% acetic acid. Molecular weight markers were purchased from Pharmacia.

Abbreviations: ALD, anterior latissimus dorsi; Tf, transferrin; CMGF, chicken muscle growth factor; MHC, myosin heavy chain; FGF, fibroblast growth factor.

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RESULTS

Heparin-Sepharose Chromatography of CMGF. Myogenic cell cultures were grown in complete medium containing embryo extract (see *Materials and Methods*). These conditions provide for optimal muscle cell growth and ultimate differentiation into myotubes (2, 3). However, when extracts (5) or purified CMGF from the ALD of adult chickens was added to such cultures there was a further increase in growth, as measured by total protein accumulation (Table 1) and by DNA synthesis and MHC accumulation (Fig. 1). The ALD extracts contain Tf (2, 3) and presumably the measured growth stimulation by the extracts could be due to Tf supplementation of total medium. Preliminary work in this laboratory has established, however, that ALD extracts are also active on rat muscle cells and since Tf stimulation of muscle growth is class specific (1), it became clear that ALD extracts contained a growth factor other than Tf (5).

To identify this growth factor we have carried out chromatography of ALD extracts on heparin-Sepharose columns. Absorption of 1 g of crude ALD extract protein to a heparin column resulted in the binding of about 50 μg of protein. The remainder appeared in the void volume. Elution of the column with 1 M NaCl released ≈40 μg of the bound protein and the remainder was eluted at 2 M NaCl. Tf did not bind to the column. Samples of total ALD extract and of the 1 and 2 M NaCl fractions were tested in myogenic cell cultures for growth-promoting activity. In this assay, cell cultures were grown in complete medium.

As shown in Table 1, total ALD extract stimulated protein accumulation 3- to 4-fold compared with control cultures.

Table 1. Activity of CMGF isolated from ALD muscle by heparin-affinity chromatography

Group	Addition to chicken myogenic cell cultures	Protein, mg per 35-mm dish
1	Control cells	30
2	ALD extract to 0.5 mg/ml before passage through heparin column	101
3	ALD extract after passage through heparin (unbound fraction, 0.5 mg/ml)	70
4	Purified chicken Tf to 20 μg/ml	73
5	Column fraction eluting at 1 M NaCl (10–40 ng/ml)	35
6	Column fraction eluting at 2 M NaCl (2–10 ng/ml)	45–60
7	Column fraction eluting at 1 M NaCl (30 ng/ml) and Tf (20 μg/ml)	80
8	Column fraction eluting at 2 M NaCl (5 ng/ml) and Tf (20 μg/ml)	103

ALD muscle extract was obtained and fractionated on heparin-Sepharose. The column was eluted batchwise, first with 1 M NaCl and then with 2 M NaCl in 10 mM Tris·HCl (pH 7.0). Ten milliliters of eluate was collected each time: 2 ml of each eluate (to be assayed for activity) was diluted with 4 ml of MEM and stored at −70°C. The remaining eluates were dialyzed against 0.05 M ammonium carbonate, lyophilized, and redissolved in distilled water for protein determination (Bradford assay, Bio-Rad) and $NaDodSO_4$/PAGE (16). CMGF effect was measured as an increase in total protein accumulation in 5-day muscle cultures containing mostly fused myotubes, as described (3). Cells were plated at 2.5×10^6 cells per 35-mm collagen-coated dish in 2 ml of 10% horse serum and 1.5% embryo extract. Cells were harvested after 5 days. Column fractions and crude extracts were added to the cells at the time of plating. Purified chicken Tf was purchased from Cappel Laboratories, Cochranville, PA, and was saturated with iron as described (2, 3). Each assay was done on four duplicate cultures and results are means ± 10%.

However, the unbound fraction also stimulated total protein accumulation, but only 2-fold, and this latter stimulation was very similar to that seen with the addition of 20 μg of chicken Tf (Table 1, groups 3 and 4). That the stimulation provided by the material in the unbound fraction was due to Tf is consistent with our earlier results showing that complete medium, even with 1.5% embryo extract, is not Tf-saturated (5). The material eluting at 1 M NaCl did not provide any growth stimulation (Table 1, group 5). However, the material eluting at 2 M NaCl stimulated total protein accumulation when applied to cell cultures in nanogram amounts (Table 1, group 6). It is this material that we call CMGF. In preliminary experiments, it also has been observed to stimulate growth of mouse muscle satellite cells as well as bovine capillary endothelial cells in cell culture. Therefore, CMGF is distinct from the class-specific Tf but works synergistically with Tf in promoting myogenesis of chicken muscle (Table 1, group 8, and Fig. 2).

Purification and Further Characterization of CMGF. The fraction eluting from heparin columns at 2 M NaCl contained a family of peptides of apparent M_rs 14,000–17,000 (Fig. 1). We have tentatively identified CMGF activity with this family of peptides. But, in addition, this fraction contained other high molecular weight components. To further purify CMGF activity, we subjected the 2 M NaCl fraction to a second round of heparin-Sepharose chromatography with elution using a gradient of 1.1–2 M NaCl. The results of this experiment are given in Fig. 1.

Material eluting from the column between 0.9 and 1.2 M NaCl contained all of the high molecular weight material that was present in the original 2 M NaCl fraction. During the gradient elution that followed, all of the CMGF activity appeared in a fraction eluting between 1.4 and 1.5 M NaCl. When this fraction was subjected to $NaDodSO_4$/PAGE analysis the result shown in Fig. 1*C* was obtained. The family of five peptides with apparent M_rs between 14,000 and 17,000 constituted the only proteins detected by Coomassie blue staining. Assuming this material to be identical to the growth factor, we calculate that a 40,000-fold purification has been achieved starting with the crude ALD extract.

Preliminary attempts to determine the isoelectric point of this family of peptides by using two-dimensional electrophoresis with isoelectric focusing in the first dimension (17) indicated that all of the CMGF bands migrated diffusely at the most basic part of the range (data not shown). The material eluting from the column at 1.4–1.5 M NaCl was also subjected to high-pressure reverse-phase liquid chromatography (HPLC) and the same five bands were seen. Unfortunately, exposure of the material to the acidic conditions and acetonitrile of HPLC resulted in an inactivation of CMGF so at the present time we cannot resolve degrees of activity among the five bands appearing either on $NaDodSO_4$/PAGE or on HPLC columns (data not shown).

When native material from the 1.4–1.5 M NaCl eluate of heparin columns was tested for activity in myogenic cell cultures the results shown in Fig. 1 *A* and *B* were obtained. DNA synthesis was stimulated by as little as 0.5 ng of CMGF per ml. We could not distinguish any additive stimulation when 5 ng of CMGF per ml was added in addition to 12 ng of FGF per ml. This CMGF activity was subject to inactivation by heat, by trypsin treatment, and by acid treatment (see Fig. 1*A*).

Purified CMGF also stimulated MHC accumulation in muscle cultures (Fig. 1*B*). In these experiments cells were grown in MEM/10% horse serum supplemented with 20 μg of chicken Tf per ml. CMGF was added on day 1 of culture. Though a single addition of CMGF induced increased cell numbers and delayed cell fusion (Fig. 2), myotube formation eventually did occur. CMGF-treated cultures showed a near doubling of MHC accumulation compared with cultures

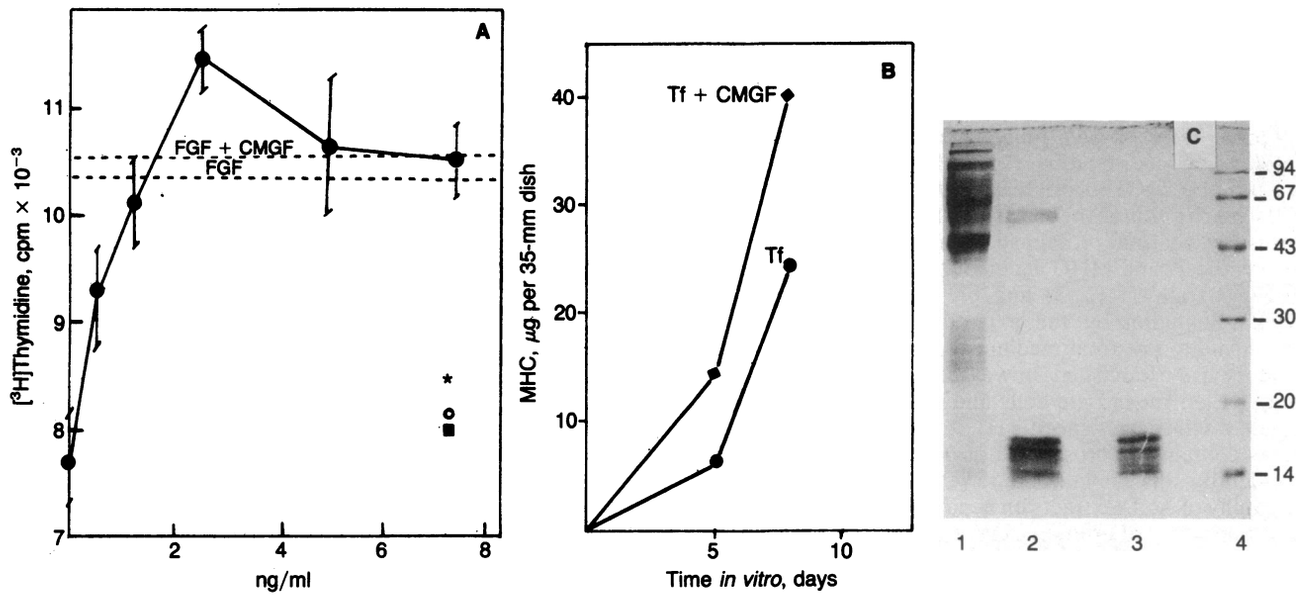

FIG. 1. CMGF purified by heparin-Sepharose chromatography stimulates DNA synthesis and MHC accumulation in myogenic cell cultures. (*A*) CMGF, twice chromatographed on heparin columns (*C*), stimulates DNA synthesis. Muscle primary cultures were obtained as described in the legend to Table 1. In this assay cells were grown in 10% horse serum and 10 μg of Tf per ml. CMGF or FGF was added to the cells at the time of plating. Dashed lines indicate the levels of stimulation in the presence of either FGF alone (12 ng/ml) or a mix of FGF and CMGF (12 ng/ml and 5 ng/ml, respectively). The asterisk (*) denotes activity of CMGF after treatment with 0.1 mg of trypsin per ml for 2 hr at 37°C; the open circle (○) denotes activity of CMGF after treatment with 0.2 M HCl for 2 hr; and the closed square (■) denotes CMGF activity after exposure to 70°C for 15 min. (*B*) Twice-chromatographed CMGF induces increased MHC accumulation. Muscle primary cultures were grown in 10% horse serum and 20 μg of Tf per ml in MEM as described (2, 3). CMGF was added at the time of cell plating. Cells were harvested after 5 and 8 days. The values shown are averaged over four dishes. (*C*) The ALD extract was first fractionated by heparin-Sepharose chromatography as described in the legend to Table 1. Lane 1, $NaDodSO_4$/PAGE of material eluting from the heparin-Sepharose column at 1 M NaCl. Lane 2, material eluting at 2 M NaCl. The 2 M NaCl fraction was collected and diluted to 0.6 M NaCl. Approximately 2 mg of this material was reapplied to a heparin-Sepharose column. A second elution cycle was started with a batch elution using 1 M NaCl followed by a gradient elution using 1–2 M NaCl. All of the high molecular weight components present in the original 2 M NaCl fraction eluted either in 1 M NaCl or in the gradient fraction eluting at 1.1 M NaCl. Lane 3, material eluting from heparin-Sepharose between 1.4 and 1.5 M NaCl. All CMGF activity also eluted between 1.4 and 1.5 M NaCl. Lane 4, migration of molecular weight markers (shown as $M_r \times 10^{-3}$).

growing in Tf alone (Fig. 1*B*). If CMGF was added after cell fusion, no further stimulation of MHC was observed (data not shown). Therefore, we conclude that the increased MHC accumulation is due to increased numbers of myogenic cells that are stimulated by CMGF and that later participate in the formation of more myotubes (Fig. 2).

The delay of onset of cell fusion induced by CMGF is illustrated in Fig. 2. After 4 days *in vitro*, chicken muscle primary cultures consisted mostly of large multinucleated myotubes (Fig. 2*A*). CMGF induced a prolongation of the proliferative phase of muscle cells since CMGF treatment resulted, after 4 days *in vitro*, in cultures consisting mostly of still actively dividing cells (Fig. 2*B*). The synergistic nature of the interaction between CMGF and Tf is shown in Fig. 2*C*. When CMGF and saturating levels of Tf were added simultaneously, 4-day cultures then were seen to consist of many actively dividing cells in addition to well-formed myotubes. Tf appears therefore to promote cell fusion, whereas CMGF appears to promote cell division and delay fusion in these cultures.

DISCUSSION

To our knowledge, a growth factor with FGF-like properties has not been isolated previously from avian muscle. The similarity of CMGF to FGF has been pointed out above. In short, the two share similar molecular weights and charge

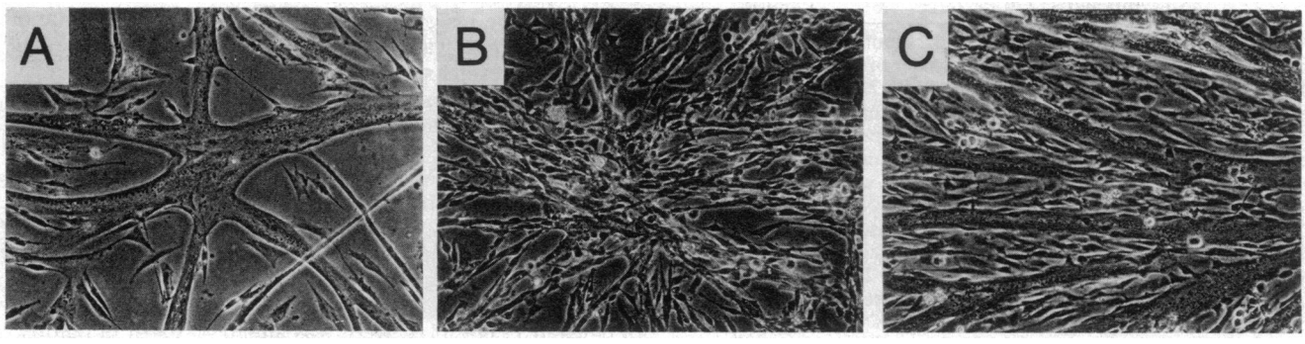

FIG. 2. Comparative effects of CMGF on growth and differentiation of muscle cells *in vitro*. Cells were grown as described for Table 1 and were photographed with phase microscopy 4 days after plating. (*A*) Control cells in complete medium. (*B*) Control cells in complete medium with 10 ng of CMGF per ml purified through two cycles of heparin-Sepharose chromatography. (*C*) Same as in b with 20 μg of chicken Tf per ml.

(18) and both inhibit muscle fusion in culture (11, 12). They both have an affinity for heparin and are subject to heat and acid inactivation (18). CMGF has no effect on either protein synthesis or MHC accumulation when applied to myotubes, and, although we are not aware of similar data for FGF, we simply point out that CMGF is a growth factor for muscle only in the sense that it stimulates myogenic cell replication.

There are a number of other growth factors in the low molecular weight range that are also immobilized by heparin at 0.6 M NaCl, irrespective of their isoelectric point. These are endothelial cell growth factor (19), chondrosarcoma-derived growth factor (20), and basic or acidic brain or pituitary FGF (18, 21, 22). On the other hand, several additional low molecular weight growth factors, such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), do not bind to heparin under conditions used by us for CMGF isolation (20). This is important because ALD muscle is somewhat more highly vascularized than is the pectoralis major muscle from which we obtained comparatively little CMGF activity (5), and it might be thought that CMGF is identical to or derived from blood PDGF. But PDGF and EGF may be eliminated as sources for CMGF simply on the basis of their lack of binding, in high salt, to heparin.

The affinity of CMGF for heparin suggests a physiological role for extracellular matrix components for regulation of muscle cell growth factors (23). We cannot say now if CMGF is synthesized *in situ* by muscle connective tissue or vascular tissue or by muscle fibers or whether it is accumulated in muscle from nerve or from more distant sites of synthesis. It is tempting to correlate this activity with the innervation pattern of the various muscles. Although the multiply innervated ALD muscle always shows relatively higher levels of Tf (2, 3) and CMGF (5) compared to a focally innervated fast muscle like the pectoralis major, the latter nevertheless contains low levels of both factors (ref. 3; unpublished data). We have found that nerve extracts contain an activity with properties very similar to CMGF (5). The nerve activity is also selectively removed by binding to heparin (unpublished data) and stimulates myogenesis in culture in a manner identical to CMGF. The ALD and other muscles therefore may accumulate CMGF from the nerve. Nerve has long been known to exert trophic influences on muscle (24, 25) and, although innervated muscle contains similar activities, denervated muscle does not (26). It is difficult to ascribe these trophic activities or influences to Tf alone. Though sciatin, a Tf-like molecule, is clearly present in the nerve (4), it is unlikely that nerve-delivered Tf could exert any precise influence given the normally high Tf levels present in blood. CMGF, however, is active in nanogram quantities and could exert such precise control.

At the same time, similarities between CMGF and FGF suggest that CMGF may also stimulate the proliferation of muscle endothelial cells and promote angiogenesis *in vivo*. Thus, its increased presence in ALD muscle could be the cause of the large number of capillaries in this tissue (27). One can only speculate now since very little is known about the molecular characteristics of FGF or certainly CMGF. By combining the existing information one can imagine the following scenario involving CMGF and Tf in muscle regeneration. Upon injury of muscle, CMGF and Tf, which are most probably included in Bischoff's crushed muscle factor (7), are released from heparin and/or other extracellular matrix binding sites in the basal lamina and in more distal interstitial structures (3, 28), stimulating the proliferation of muscle satellite cells and cells of the vascular endothelium. Release of basal lamina bound factors could be mediated by lymphocytic invasion of muscle through the inflammatory response and attendant degradation of heparin sulfate basal lamina components (29). The regenerating muscle meanwhile is known to secrete substances that promote neurite outgrowth (30) and the various components of muscle regeneration would thus be coordinated.

Further work is clearly required to characterize CMGF and its putative relationship to or identity with FGF and other growth factors in both avian and mammalian organisms. The mechanism by which such a potent growth factor is accumulated by muscle tissue and the relationship of this accumulation to regulation of muscle growth and regeneration also await further study.

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