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Biochemistry

Isolation of a cationic polypeptide from human serum that stimulates proliferation of 3T3 cells

(Balb/c-3T3/growth factors/somatomedin/insulin-like activity)

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ABSTRACT **A basic polypeptide that stimulates DNA synthesis and cell division in confluent populations of mouse Balb/c-3T3 cells has been isolated from whole human serum, and has been separated from the heterogenous group of molecules with insulin-like activity. This highly purified basic polypeptide has a molecular weight of 1.3×10^4 and an isoelectric point of 9.7. Approximately 10^7 polypeptide molecules in the growth medium allow the replication of one density-inhibited cell.**

The population density of fibroblasts in culture is controlled by the concentration of serum that is present in the growth medium. Mouse Balb/c-3T3 cells are subject to density-dependent inhibition of cell division and cease to replicate when confluence is attained (1). These cells become arrested in the G_1 phase of the growth cycle prior to DNA synthesis. The addition of whole serum stimulates these cells to progress through the S phase and mitosis, and the population attains a higher saturation density (2). The growth of cells transformed by oncogenic viruses is not regulated by density-dependent inhibition of cell division, and many viral-transformants grow to a high saturation density, in the presence of a low concentration of serum (3).

Whole serum contains several factors needed to support the growth of Balb/c-3T3, while other distinct factors allow the growth of viral-transformants (4). Several serum factors capable of stimulating the proliferation of Balb/c-3T3 have been partially purified (4). Insulin (5–7) and glucocorticoids (8) may potentiate the growth-stimulating effect of these factors. Both insulin and serum have a pleotypic effect on 3T3 cells, inducing RNA and protein synthesis and preventing protein degradation (9). These hormones are not needed for the maintenance of the pleotypic effect in transformed cells (9). Insulin and serum proteins with insulin-like activity may stimulate the proliferation of chick cells (10, 11). Scher *et al.* (12), however, have demonstrated that the serum factors with insulin-like activity are heat labile and distinct from those that stimulate the proliferation of Balb/c-3T3.

We now describe the isolation of a cationic polypeptide from whole human serum that stimulates confluent populations of Balb/c-3T3 to proliferate. This highly purified polypeptide is distinct from the heterogenous group of molecules with insulin-like activity.

MATERIALS AND METHODS

Ion-Exchange Chromatography. Pooled human serum 1.2 liter) was mixed with an equal volume of wet Dowex-50W X 8 resin (Na+ form) (13). The mixture was stirred gently for 20 min; the serum was then decanted and subjected twice to the same treatment with fresh resin. The resin from the three consecutive serum treatments was placed into a column (8 × 97 cm) and washed with 0.15 M NaCl; the adsorbed proteins were eluted from the resin with NH_4OH (0.02 N), as described (13). The eluate was condensed by ultrafiltration at 2° using Amicon Diaflow UM-10 membranes. The solution inside the ultrafiltration cell containing the growth factors and insulin-like activity was lyophilized. One liter of serum yielded about 80 mg of protein.

Gel Filtration. Lyophilized preparations obtained by ion-exchange chromatography were dissolved in cold H_2O (80 mg of protein in 5 ml) and dialyzed overnight at 2° against 200 volumes of 0.15 M NaCl. The dialyzed sample was applied to a Sephadex G-100 column (bed volume—970 ml) in 0.1 M borate buffer, pH 8.0 at 2°. Elution was carried out with borate buffer at a rate of 22 ml/hr. Ten milliliter fractions were collected, and fractions corresponding to various molecular sizes were pooled and lyophilized. Each pooled fraction was dissolved in 10 ml of 0.15 M NaCl. Aliquots of 0.2 ml were added to 0.9 ml of depleted media containing [^{3}H] thymidine to test for DNA synthesis. For the assay of insulin-like activity, samples from the pooled fractions were injected into rats (1 ml per rat).

Pooled Sephadex fractions (300 mg of protein) from about 21 liters of serum corresponding to molecular weights of 6,000–70,000 were dissolved in H_2O, condensed by ultrafiltration using Amicon Diaflow UM-10 membranes, and dialyzed against 0.15 M NaCl. The dialyzed preparation (8 ml) representing 21 liters of original serum was re-chromatographed on the Sephadex G-100 column and fractions were collected, pooled, and lyophilized. The final volume of the pooled fractions was 10 ml. Samples from each pooled fraction were diluted 1:10 with 0.15 M NaCl and assayed for insulin-like activity and the stimulation of DNA synthesis as described.

Preparative Isoelectric Focusing on Polyacrylamide Gels. Sephadex fractions from the 21 liter serum run were subjected to isoelectric focusing in 20% sucrose containing 1% LKB ampholytes (0.9% Ampholines pH 3–10; 0.1% pH 9–11). The preparations, with a methyl red marker, were applied to a 5% polyacrylamide gel at 2°, which had been prerun for 30 min. The gel for isoelectric focusing was contained in four separate vertical cylinders (total length 10 cm, diameter 2.1 cm) separated by liquid interphases (D. Stathakos, in preparation). The formation of the pH gradient was followed by measuring the pH of samples withdrawn from

Abbreviation: pI, isoelectric point.

these liquid layers. The potential was kept below 180 V to avoid heating. The anolyte and catholyte were 0.01 M H_3PO_4 and 0.02 M NaOH, respectively. After the run, the gel was sectioned at 4 mm intervals and the ampholyte was eluted for determination of pH by mild stirring for 60 min in H_2O. The protein from the sliced gels was eluted with 1 M NaCl (10 ml/4 mm section) and dialyzed against 0.15 M NaCl. Samples from the dialyzed fractions were assayed for the stimulation of DNA synthesis and insulin-like activity as described.

Incubation with mercaptoethanol. Preparations were incubated for 1 hr at 25° with an equal volume of 20 mM 2-mercaptoethanol in 20 mM Tris·HCl buffer (pH 7.4). Preparations lacking mercaptoethanol were incubated in parallel. The samples were then dialyzed extensively against 0.15 M NaCl at 2° to remove the reducing agent.

Incubation with Trypsin and Chymotrypsin. The Dowex-adsorbed serum fraction (protein concentration—2 mg/ml) was buffered with 0.01 M Tris·HCl (pH 7.4) and incubated for 6 hr at 37° with 1 mg/ml of trypsin or chymotrypsin. The serum fractions without enzymes and enzymes alone were incubated in parallel. After incubation, all samples were heated to 100° for 20 min (12) to inactivate the proteolytic enzymes. They were then diluted in depleted medium and added to the cells to stimulate DNA synthesis.

Assay for DNA Synthesis and Cell Division. Balb/c-3T3 (clone A31) cells were maintained on Falcon microtiter wells (area 0.3 cm^2) in Dulbecco's modification of Eagle's medium containing 4.5 g of glucose per liter and 2% calf serum (Colorado Serum Co.). Each well contained approximately 1.3×10^4 cells at confluence. The human serum fractions were added to medium containing 5 μCi/ml [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) and 2% calf serum that had been previously depleted on Balb/c-3T3. The volume of medium added to each microtiter well was 0.2 ml. About 48 hr later the cells were fixed and washed three times with 5% trichloroacetic acid. The percentage of cells which synthesized DNA was found using autoradiography, while the total number of cells present was determined with the aid of a microscopic grid (12). More than 200 cells were counted for each determination, while approximately 1000 were scanned.

Assay of Insulin-Like Activity. Insulin-like activity was measured by the conversion of glucose to glycogen by the diaphragm of the rat (14). The samples to be assayed, or crystalline insulin standards, were injected intraperitoneally into intact rats together with [*U*-^{14}C]glucose (3.3 Ci/mol). Two hours later, the rats were lightly narcotized with 50% CO_2 and decapitated. Their two hemidiaphragms were removed, weighed, and hydrolyzed in 30% KOH (w/v) at 100°. The glycogen was precipitated by the addition of Na_2SO_4 to 12 mM and absolute ethanol to a final concentration of 70% (v/v), separated by centrifugation, and analyzed for radioactivity. Each sample was assayed in five rats. The values given represent the mean of five parallel determinations.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Polyacrylamide (12%) gel electrophoresis in 2% sodium dodecyl sulfate was carried out in a multiphasic buffer system (N. Catsimpoolas, in preparation) with molecular markers of cytochrome *c*, lysozyme, and myoglobin. Molecular weight estimation was performed by a computer program similar to that described previously (15) for the analysis of polyacrylamide gel electrophoresis data.

Other Methods. Protein concentration was determined by the method of Lowry *et al.* (16) with human serum albumin as the standard.

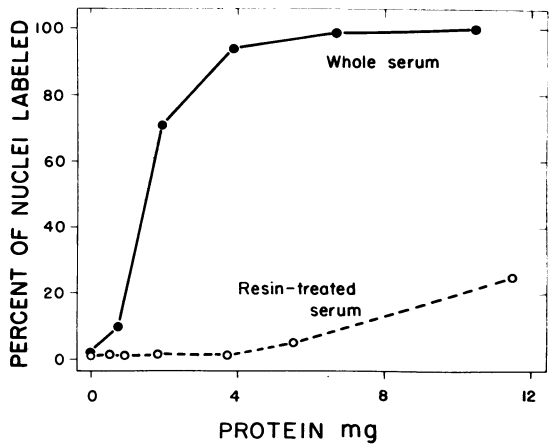

FIG. 1. DNA synthesis induced by whole human serum before and after treatment with the Dowex-50 cationic exchange resin.

RESULTS

Ion-Exchange Chromatography. The confluent Balb/c-3T3 cells used in all experiments were maintained in medium containing whole calf serum that had been depleted (1) of growth factors by previous exposure to Balb/c-3T3. Confluent Balb/c-3T3 cells maintained in this medium do not proliferate, but can be stimulated to divide by the addition of whole human serum. The factor(s) in human serum that induce these cells to proliferate can be almost completely removed by a Dowex resin (Fig. 1). These factors can be eluted from the Dowex resin and induce DNA synthesis and mitosis in confluent Balb/c-3T3 cells. Dowex treatment of

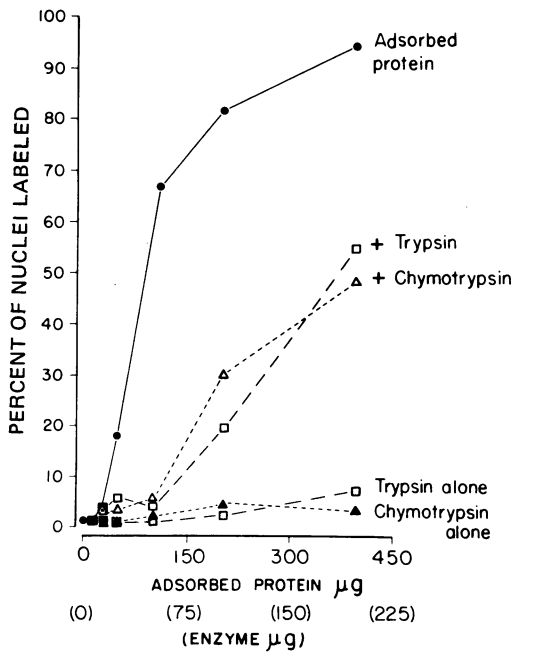

FIG. 2. Effect of trypsin and chymotrypsin on the Dowex-resin-adsorbed proteins that stimulate DNA synthesis. After treatment with trypsin (+ trypsin) or chymotrypsin (+ chymotrypsin) all samples, including the control samples, were heated to 100° to inactivate the proteolytic enzymes.

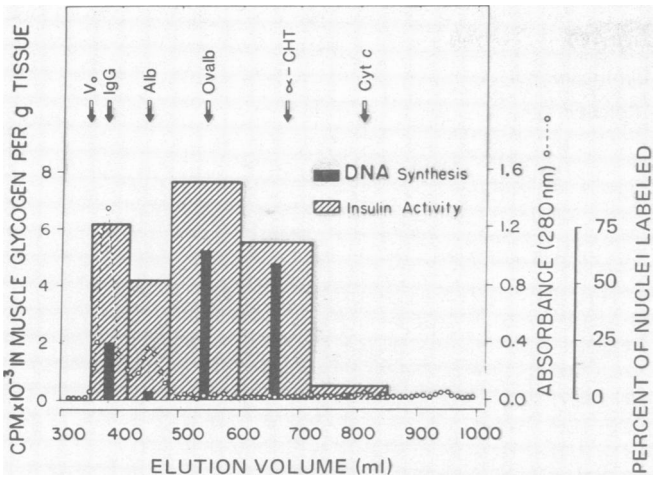

FIG. 3. Sephadex G-100 chromatography of the proteins eluted from the Dowex resin. DNA synthesis was determined by autoradiography, while the insulin-like activity was assayed by the conversion of glucose to glycogen. For the purpose of clarity, the unstimulated control values for DNA synthesis and glycogen synthesis were subtracted from this and subsequent figures. Elution positions are: V_0, void volume; IgG, immunoglobulin G; Alb, serum albumin; Ovalb, ovalbumin; α-CHT, α-chymotrypsin; Cyt *c*, cytochrome *c*.

whole human serum routinely allowed the recovery of 8 mg of protein from 7 g of whole serum protein. The fraction eluted from the Dowex resin contained the serum factors that induced DNA synthesis and mitosis in confluent Balb/c-3T3 cells. Approximately 100 μg of the adsorbed protein stimulated 50% of the Balb/c-3T3 cells to enter the S phase (Fig. 2). Although the Dowex eluate stimulated the proliferation of cells maintained in serum-depleted medium, it did not allow the survival of cells maintained in the absence of serum (12).

The serum fraction eluted from the Dowex resin also contained the factors with insulin-like activity (12). These factors have the physiologic activity of insulin and cause muscle cells to take up extracellular glucose and convert it to intracellular glycogen. No free insulin could be detected in these preparations using a radioimmunoassay.

To demonstrate that the partially purified cell division-stimulating factors are proteins, they were subjected to proteolytic degradation with trypsin or chymotrypsin. Proteolytic enzymes themselves, however, stimulate cell division (17). Thus, it was necessary to inactivate these enzymes before applying the treated serum samples to cells. After treatment with trypsin or chymotrypsin, the samples were heated to 100° for 20 min to selectively destroy proteolytic activity. Heating does not affect the ability of the serum fractions to stimulate the proliferation of Balb/c-3T3 (12). Incubation of the Dowex-adsorbed protein with trypsin or chymotrypsin caused a loss in the ability of these preparations to stimulate DNA synthesis (Fig. 2). Although 100 μg of the untreated Dowex-adsorbed proteins stimulated 50% of the confluent cells to enter the S phase, 400 μg of the enzyme-treated preparations were required to achieve the same degree of stimulation. Thus, each proteolytic enzyme caused a 75% loss in the ability of this preparation to stimulate DNA synthesis. Reduction with mercaptoethanol completely abolished the ability of this preparation to stimulate cellular proliferation.

Sephadex G-100 Chromatography. Gel filtration allowed a further purification of the factors that stimulate DNA synthesis. The Dowex-adsorbed proteins (80 mg) were applied to a Sephadex G-100 column and fractions were assayed for the stimulation of DNA synthesis and for insulin-like activity. The majority of both activities eluted together (Fig. 3) with an elution volume of 350–725 ml, while the majority of the protein eluted before 475 ml. Thus, the fractions with the highest specific activity eluted after 475 ml. The fractions with high specific activity from several separate Sephadex runs (elution volume 475–850 ml) were pooled and 300 mg of protein representing 21 liters of human serum were reapplied to a Sephadex G-100 column. Fig. 4 shows that the factor(s) that stimulate DNA synthesis were present in Sephadex fraction IV (elution volume 550–630 ml), and in Sephadex fraction V (elution volume 630–775 ml). Proteins with insulin-like activity, however, were present only in fraction IV. Between 20 and 30 μg of protein from each of

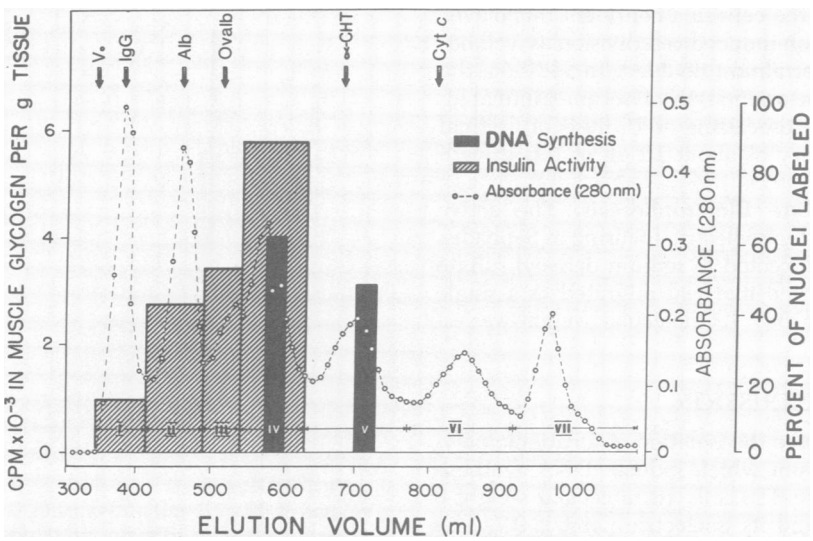

FIG. 4. Sephadex G-100 rechromatography of pooled fractions (elution volume 475–840 ml) from several initial Sephadex runs. About 300 mg of protein from 21 liters of serum were rechromatographed and assayed for the stimulation of DNA synthesis and insulin-like activity.

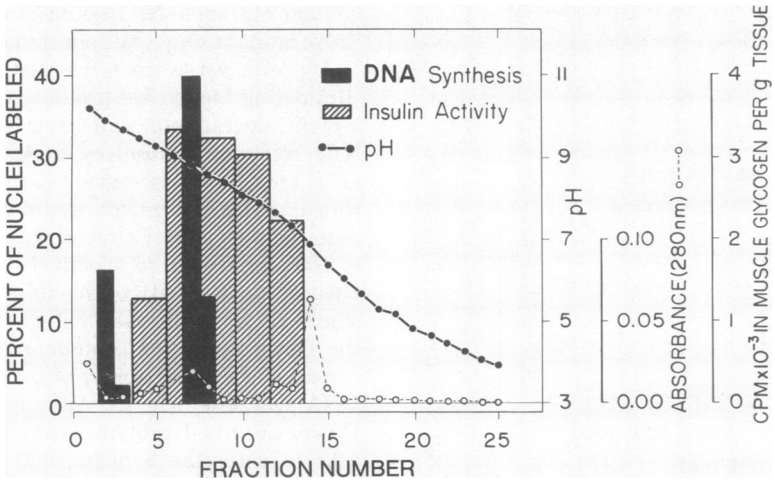

FIG. 5. Preparative isoelectric focusing of fraction IV (elution volume of 550–630 ml) of Sephadex G-100 rechromatography (Fig. 4). Approximately 2 mg of protein were applied to the gel. Each fraction was tested for the stimulation of DNA synthesis, while adjacent fractions were pooled to assay for insulin-like activity.

these fractions was required to induce DNA synthesis in 50% of the Balb/c-3T3 cells.

Isoelectric Focusing. Preparative isoelectric focusing of Sephadex fraction IV (2 mg of protein applied representing 2 liters of original serum) resolved two sharp peaks that stimulated DNA synthesis (pI of 9.7–9.9 and 8.6–8.8), while the insulin-like activity was more heterogenous (pI of 7.3–9.4) (Fig. 5). Thus, a distinct factor that stimulated DNA synthesis has been separated from the factors with insulin-like activity. Although one growth factor is found among the molecules with insulin-like activity, the majority of the polypeptides with insulin-like activity do not stimulate DNA synthesis.

Isoelectric focusing of Sephadex fraction V (10 mg of protein applied representing 10 liters of original serum) revealed that the activity stimulating DNA synthesis again focused in an area corresponding to pH 9.6–9.8 (Fig. 6). All isoelectric focusing fractions were now devoid of insulin-like activity, a finding consistent with the absence of such activity in the original Sephadex fraction. About 8 ng protein of the activity fraction with a pI of 9.7 was sufficient to induce DNA synthesis in 87% of the cells in a confluent monolayer of Balb/c-3T3. This fraction induced cell division as well because the total cell number almost doubled. In addition, the cells assumed the random orientation of serum-stimulated, or transformed, cells. The processing of 10 liters of original serum allowed the recovery of about 10 μg of this growth factor.

Sodium Dodecyl Sulfate Electrophoresis. The active fraction (pI 9.7) obtained by isoelectric focusing of Sephadex fraction V (Fig. 6) was subjected to sodium dodecyl sulfate electrophoresis. The purified material migrated as a single component with an R_F of .845. Its molecular weight was estimated to be 13,000.

DISCUSSION

The present studies describe the isolation of a cationic polypeptide from human serum which induces DNA synthesis and cell division in Balb/c-3T3 cells. Its molecular weight is estimated to be 13,000 and its isoelectric point is 9.7. This basic polypeptide is free of insulin-like activity. About 8 ng of this highly purified polypeptide allowed 10^4 Balb/c-3T3 cells maintained in a serum-depleted medium to proliferate. Under these conditions, approximately 10^7 molecules in 0.2 ml of the growth medium stimulated the replication of one density-inhibited cell.

The human serum growth factor is a cationic polypeptide that induces cell division. This effect appears to be quite specific. Other basic molecules including DEAE-dextran, Polybrene, protamine-insulin, and putrescine do not stimulate the growth of confluent Balb/c-3T3 (data not shown).

In addition to the basic polypeptide our studies demonstrate the presence of other cationic substances in the serum preparations, which also induce DNA synthesis and cell division. Their isoelectric point is between 8.6 and 8.8. Their relationship to the basic polypeptide described here is not known. They may represent unrelated cationic proteins capable of inducing DNA synthesis and cell division, aggre-

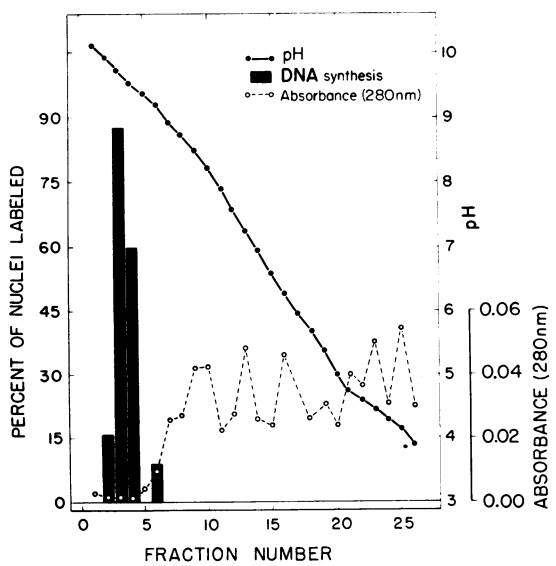

FIG. 6. Preparative isoelectric focusing of fraction V (elution volume of 630–775 ml) of Sephadex G-100 rechromatography (Fig. 4). Approximately 10 mg of protein were applied to the gel. Each eluted fraction was diluted 1:5 before being tested for DNA synthesis or insulin-like activity. None of the fractions exhibited insulin-like activity.

gates of the basic polypeptide, or complexes with other serum proteins altering the isoelectric point.

Several partially purified factors have been isolated from serum that stimulate the proliferation of cells in culture (5, 10, 18, 19). In addition, serum factors have been described that stimulate the growth of cartilage. These factors, often termed somatomedins (19, 20), have not been separated from the molecules with insulin-like activity. Our present studies indicate that at least one of the serum growth factors is distinct and can be separated from the heterogenous group of serum polypeptides exhibiting the insulin-like activity.

Recently, Gospodarowicz (21) has isolated a polypeptide from bovine pituitary glands that stimulates the growth of Balb/c-3T3. This growth factor is a basic polypeptide with a molecular weight of 13,300 (22). These properties are similar to those of the circulating basic polypeptide described herein. This circulating basic polypeptide may prove to be of pituitary origin.

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