Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 874-880

A PARTIALLY PURIFIED SERUM FRACTION SYNERGISTICALLY ENHANCES THE MITOGENIC ACTIVITY OF EPIDERMAL GROWTH FACTOR AND INSULIN IN QUIESCENT CULTURES OF 3T3 CELLS

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Received June 7,1978

SUMMARY

Epidermal growth factor and insulin need a low concentration of serum to effectively stimulate quiescent 3T3 cells into DNA synthesis. We partially purify a polypeptide component of serum which has no activity by itself but which acts synergistically with epidermal growth factor and insulin to stimulate cultures of 3T3 cells into DNA synthesis as effectively as whole serum. The active fraction is separated from serum by gel chromatography on Sephadex G-100, under acid dissociating conditions, and chromatographs with a molecular weight of 18,000 daltons.

We suggest as a general technique, the use of pure growth factors to assay for growth promoting fractions from serum or other sources. Fractions which are not mitogenic by themselves can be detected when assayed together with their complementary pure factors.

Mouse epidermal growth factor (EGF)* a pure polypeptide of 6045 daltons (1) promotes growth of a variety of cell types (2-4) including fibroblasts in cell culture (5-8). In conjunction with insulin, EGF stimulates DNA synthesis in quiescent cultures of 3T6 cells in completely serum-free medium (9,10). However, in other cell types the growth promoting activity of EGF is largely dependent on the presence of a low concentration of serum in the nutrient medium. This requirement for serum has been documented in cultures of epithelial cells (11), human diploid fibroblasts (5,8,12) and mouse 3T3 cells (7) and cannot be replaced by serum albumin, insulin, hydrocortisone or testosterone (5,8,12). Furthermore, addition of serum does not affect the binding of EGF to surface receptors (13). The role of serum in the cellular response to EGF remains unresolved.

In the present paper we report the partial purification of a polypeptide from serum

*Abbreviations: EGF: Epidermal Growth Factor. NSILA: Non-suppressible Insulin-like Activity. MSA: Multiplication Stimulating Activity. DEM: Dulbecco's Modified Eagle's Medium. DEM/W: 1:1 mixture of DEM and Waymouth medium. P.P: Protein peak.

0006-291X/78/0833-0874$01.00/0

which, when added with insulin potentiates the mitogenic activity of EGF in quiescent cultures of 3T3 cells. The two growth factors, when combined with the partially purified serum fraction are as potent as whole serum in stimulating DNA synthesis.

MATERIALS AND METHODS

Cell culture. Swiss mouse 3T3 cells, propagated as previously described (14), were subcultured into 33 mm Nunc dishes in 10% serum and grown to confluence in this medium. All assays of growth promoting activity were performed on such cultures. The cultures of 3T3 cells used in this study were arrested in the G_1/G_0 phase of the cell cycle. After exposure to [^{3}H]thymidine for 40 hr, less than 1% of the nuclei in the culture became radioactively labelled.

Assays of growth promoting activity. All determinations of DNA synthesis were performed in a 1:1 mixture of DEM and Waymouth medium (DEM/W). The cultures were washed twice with DEM to remove residual serum immediately prior to assaying. For determinations of DNA synthesis, the medium (2 ml) contained either 0.2 μM (5 μCi ml^{-1}) or 2.5 μM (0.5 μCi ml^{-1}) [^{3}H] thymidine for autoradiography and incorporation into acid precipitable DNA respectively. Incubation with fractions and label were for 40 hr, unless stated otherwise.

Fractionation of serum. Twenty-five ml of fetal bovine serum, adjusted to pH 2, were fractionated on a column (95 x 5 cm) of Sephadex G-100 in 0.1 M NaCl, 0.01 N HCl, pH 2, at 4°. Preliminary experiments showed that gel filtration under such conditions completely separated the growth promoting activity from the bulk of the serum protein (data not shown). The fractions eluting after the main protein peak were pooled, dialyzed against 0.02 N HCOOH and lyophilized. To provide sufficient material the lyophilisates from five chromatographic separations were pooled (total volume 30 ml) and rechromatographed on the same column. Approximately 9 ml fractions were collected. The column was calibrated for molecular weight determinations with the following markers: Blue dextran (void volume); ovalbumin (42,000); myoglobin (16,900); cytochrome C (13,400) and insulin (6,500). The protein concentrations of various solutions were estimated by the procedure of Lowry (15).

Materials. Mouse epidermal growth factor was generously supply by Dr. S. Cohen. Bovine insulin (26 I.U. mg^{-1}) was obtained from Sigma. [^{3}H] thymidine was from the Radiochemical Centre, Amersham. The serum used was fetal bovine (Flow Laboratories). Sephadex G-100 and the chromatographic column were from Pharmacia. All other materials used were of reagent grade.

RESULTS

Neither EGF (1 ng ml^{-1}) nor insulin (1 μg ml^{-1}) added to quiescent 3T3 cells arrested in the G_1/G_0 phase of the cell cycle stimulate DNA synthesis (Fig. 1). When both are added together, only a small proportion of the cells are labelled; the labelling index varied from 5-20% in different experiments. However, when the hormones are tested at the same concentrations in the presence of low concentrations of serum, they become potent stimulators of DNA synthesis. Figure 1 shows that 3% serum and the EGF-insulin combination give labelling indexes of 2% and 20%, respectively. Combined,

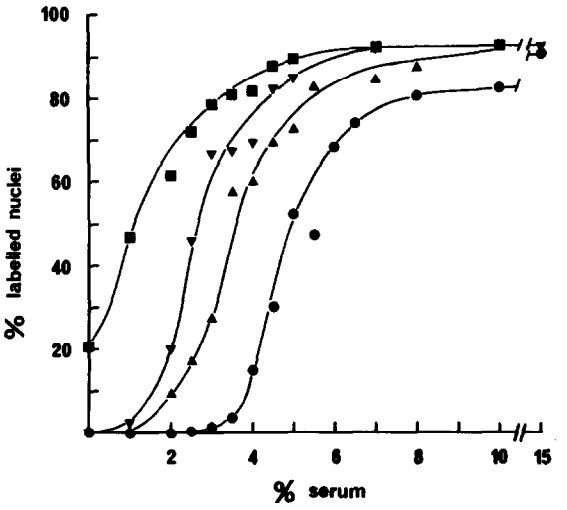


Fig. 1. Dependence of DNA synthesis on the concentration of serum in the medium, in the absence or presence of insulin, EGF or both. Additions were made to quiescent cultures of 3T3 cells as follows: (●) no additions; (▼) 1 μg ml^{-1} insulin; (▲) 2 ng ml^{-1} EGF; (■) 2 ng ml^{-1} EGF + 1 μg ml^{-1} insulin. The proportion of the cells in DNA synthesis was assessed by autoradiography. All other experimental procedures were as described under Materials and Methods.

they stimulate 80% of the culture. It is apparent that EGF and insulin act synergistically with an undefined component from serum. It has been reported that the serum required by cultured human fibroblasts for effective stimulation by EGF can be partially replaced by ascorbic acid (0.5-10 μg ml^{-1}) (8,12). We found that 9 μg ml^{-1} of the vitamin (the level in DEM/W medium) has no enhancing effect on the activity of EGF in 3T3 cells.

In order to isolate the serum fraction(s) that specifically potentiates the growth promoting activity of EGF and insulin, serum was fractionated on columns of Sephadex G-100 as described in Materials and Methods, and the resulting fractions were assayed in quiescent cultures of 3T3 cells in the absence and presence of EGF and insulin (Fig. 2). Negligible growth promoting activity is detected in the absence of the peptides. In contrast, two peaks of stimulatory activity are revealed when EGF and insulin are added to the assay medium. A prominent peak, termed A, elutes at a K_{av} corresponding to a mean molecular weight of 18,000 daltons, and a less active peak, termed B, elutes at a position corresponding to a mean molecular weight of 14,000 daltons. The low molecular weight region of the column (termed peak C) which probably contains some of the family of growth promoting peptides such as MSA, NSILA and the somatomedins which elute in this region of the column (see 16 for refs.) has little activity in 3T3 cells.

To further investigate the potency and specificity of the serum fractions, the

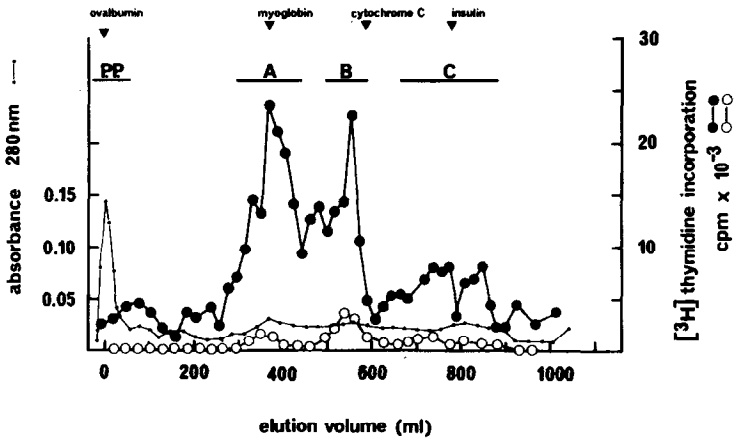
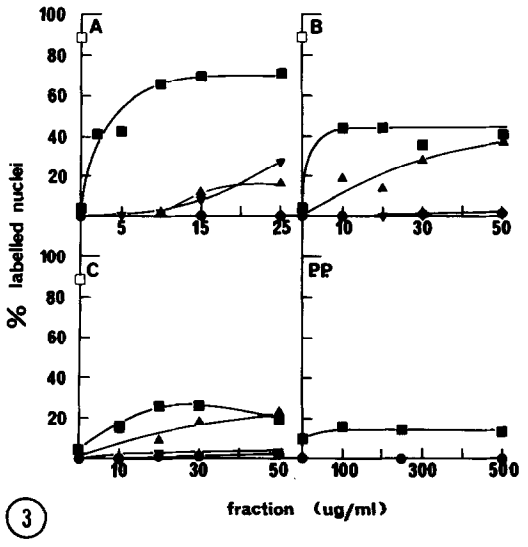


Fig. 2. Rechromatography of pooled fractions from several initial Sephadex G-100 separations. Gel chromatography was conducted as described under Materials and Methods. Aliquots of 0.3 ml were assayed in 3T3 cells in the absence (○) and in the presence (●) of EGF (1 ng ml^{-1}) and insulin (0.5 μg ml^{-1}). Arrow heads indicate mean elution volumes of molecular weight markers, chromatographed separately on the same column. Horizontal bars indicate the regions of the column which were pooled and concentrated.

fractions representing peaks A, B and C were pooled, dialyzed against 20 mM formic acid, lyophilized and tested in cultures at different concentrations. Figure 3 shows that none of the fractions display any activity by themselves. Fraction A slightly enhances the mitogenic activity of the individual growth factors. However, when both EGF and insulin are present, this fraction causes a very marked potentiation of their activity. Fraction A gives a half maximal stimulation of the cultures at a protein concentration of 3.3 μg ml^{-1} (Fig. 3). Serum with added pure factors, gives such a stimulation at a concentration of 1.4% (approximately 0.84 mg ml^{-1} protein) (Fig. 1). This represents approximately a 250-fold purification of growth promoting activity from serum. However, analysis by disc gel electrophoresis shows that the fraction contains a heterogeneous mixture of many different components. None of the other fractions added at different concentrations are as effective as fraction A. The main protein peak (P.P.), which elutes at the void volume, is totally inactive.

The conclusions drawn from the dose-responses presented in Figure 3 are further substantiated by experiments in which the effects of the various serum fractions were assessed as a function of time. The fractions, added at saturating concentrations, in combination with EGF and insulin, stimulate cells into DNA synthesis after a lag period which is identical to that given by serum (Fig. 4). The rate of entry into the S phase and the proportion of the culture stimulated vary according to the mitogenic potencies of the fractions, a finding consistent with the data presented in Figures 2 and 3.



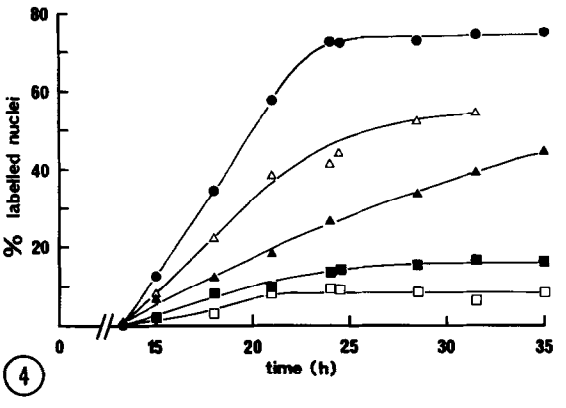


Fig. 3. Dependence of DNA synthesis on the concentration of the partially purified fractions, in the absence and in the presence of EGF and insulin. The growth-promoting activity of the fractions was assessed by autoradiography. The panels A, B, C and P.P. refer to fractions obtained by pooling and concentrating the regions of the column illustrated in Fig. 2. Each fraction was tested as follows: (●) no additions; (▲) 2 ng ml^{-1} EGF; (▼) 2 µg ml^{-1} insulin; (■) 2 ng ml^{-1} EGF + 2 µg ml^{-1} insulin. The medium concentration of the fractions represents µg protein ml^{-1}. Data for P.P. is from a separate experiment. The effect of 10% serum (□) is shown for comparison.

Fig. 4. Stimulation of DNA synthesis by the partially purified serum fractions in the presence of EGF and insulin, measured as a function of time. Factors and fractions were added at 0 hr. At the times indicated, dishes from each group were fixed and processed for autoradiography. The cultures were exposed to 2 ng ml^{-1} EGF + 2 µg ml^{-1} insulin (□) or to these hormones plus 40 µg ml^{-1} fraction A (●), 50 µg ml^{-1} fraction C (▲) or to 250 µg ml^{-1} of the protein peak (■). The effect of medium containing 5% serum (△) in this experiment is shown for comparison.

In all the above experiments similar results were obtained when (^{3}H) thymidine incorporation into acid precipitable material was measured instead of the labelling index. Three different batches of serum, fractionated by the same procedure gave very similar patterns of peaks with growth promoting activity. Preliminary characterization of fraction A shows that its activity is probably due to a polypeptide, because it is destroyed by trypsin (2.5 mg ml^{-1} 3 hr, 37°). The activity is stable to boiling (20 min, pH 2.0 and pH 7.4) and is not decreased by disulfide reduction (0.1 M mercaptoethanol, 3 hr, 37°). The fraction also has biological effects on quiescent 3T3 cells; both uridine and deoxyglucose uptake (measured as described in reference 17) are stimulated 30 minutes after adding the fraction (unpublished data).

DISCUSSION

Recent studies have shown that growth promoting factors change the rate of cell proliferation in a synergistic manner (17-20). Thus, in theory, some growth promoting materials will only show activity when they are assayed with their complementary factors. Our findings provide a direct substantiation of this possibility and suggest a strategy for the assay of growth promoting activity in fractions from serum or other sources. In this paper we describe the partial purification of a serum component which is totally inactive by itself, but which, when combined with EGF and insulin, stimulates quiescent cultures of 3T3 cells as effectively as whole serum.

The nature of the component in the fraction which acts synergistically with EGF and insulin is not known. An arginine esterase (m.wt. 29,300 (21) or 26,600 (22)) which binds EGF has been reported to potentiate approximately two-fold the mitogenic activity of EGF in cultured human fibroblasts in the presence of serum or ascorbic acid (8). It is unlikely that the activity of fraction A is due to this enzyme because the fraction has a lower apparent molecular weight (Fig. 2), it requires EGF and insulin but not whole serum or ascorbic acid for its activity, and it potentiates up to fifteen-fold the activity of EGF and insulin (Fig. 3). Since the fraction chromatographs with a molecular weight corresponding to approximately 18,000 daltons under conditions of molecular dissociation (23), and is assayed in the presence of a saturating concentration of insulin, it is unlikely to be one of the small polypeptides with insulin-like and growth promoting activity, such as NSILA, MSA or the somatomedins (16) previously isolated from serum. The fibroblast derived growth factor (FDGF) has a comparable m.wt. but is isolated from medium conditioned by SV40 BHK cells (17). Finally, disulfide reduction, which abolishes the growth promoting activity of NSILA (24), MSA (25), and the cationic serum factor isolated by Antoniades et al (26), does not reduce the activity of fraction A. The potential biological significance of the active component of fraction A warrants further experimental work to elucidate its nature and mechanism of action.

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