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Transforming growth factors: Isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction

(sarcoma growth factors/cell transformation/soft agar growth/murine sarcoma virus)

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ABSTRACT **Polypeptides characterized by their ability to confer a transformed phenotype on an untransformed indicator cell have been isolated directly from tumor cells growing both in culture and in the animal, by using an acid/ethanol extraction procedure. Assay of these polypeptides is based on their ability to induce normal rat kidney fibroblasts to form colonies in soft agar. Peptides from murine sarcoma virus-transformed mouse 3T3 cells grown in culture had the highest specific activity in this assay; peptides from sarcomas produced from these cells or from chemically induced transplantable bladder carcinomas of mice were one-third as active; and peptides from a chemically induced rat tracheal carcinoma had only one-tenth the activity. Treatment with either trypsin or dithiothreitol destroyed the activity of all of these materials. The properties of these intracellular polypeptides from both virally and chemically transformed cells are similar to those described for the sarcoma growth factors (SGFs) previously isolated from the conditioned medium of sarcoma virus-transformed mouse 3T3 cells, suggesting the definition of a class of transforming growth factors common to tumor cells of different origins. The transforming peptides from the cultured sarcoma virus-infected cells were separated by gel filtration into two fractions of apparent molecular weight 7000 and 10,000. The major fraction at molecular weight 7000 represented approximately 0.1% of the original cell protein and had a specific activity 50 times that of the original acid/ethanol extract.**

A family of heat- and acid-stable transforming polypeptides, termed sarcoma growth factors (SGFs), has recently been isolated from the conditioned medium of Moloney murine sarcoma virus (MuSV)-infected mouse 3T3 cells by De Larco and Todaro (1). The SGFs have been shown to compete with epidermal growth factor (EGF) for available membrane receptors, yet they do not crossreact with antibodies to EGF, and their biological activity is distinct from that of EGF. Addition of these SGFs to the culture medium of normal cells results in a rapid and reversible change of cellular morphology to that characteristic of the transformed state. Associated with the morphological changes are other indices of transformation such as the ability of the treated cells to overgrow in monolayer cell culture and to form progressively growing colonies in soft agar. Thus these polypeptides have the property of reversibly conferring the transformed phenotype on normal cells *in vitro*, and, in this sense, can tentatively be considered proximate effectors of the malignant phenotype (1).

For the purposes of purification of these polypeptides, it was desired to extract them directly from tumor cells, rather than from conditioned medium. It was also important to ascertain whether the SGFs were unique to MuSV transformation, or whether they were representative of a class of proteins with similar properties, which we shall call transforming growth factors (TGFs) (2). We now report finding TGFs in cultured MuSV-transformed 3T3 cells and in sarcomas produced by inoculation of these cells into athymic mice. Moreover, TGFs of specific activity approximately equal to that of the sarcomas have been isolated from chemically induced bladder carcinomas. These TGFs have been extracted from the cells with acid/ethanol, a procedure that has previously been applied to the extraction from organs and from blood of such biologically active polypeptides as insulin (3, 4), glucagon (5), insulin-like growth factor (6), the somatomedins (7), and secretin (8). The demonstrated application of this method to the finding of a class of acid-stable TGFs from cells transformed by either a virus or chemicals brings a unifying concept to the mechanism of carcinogenesis.

MATERIALS AND METHODS

MuSV-Transformed 3T3 Cells. The Moloney MuSV-transformed 3T3 cell line 3B11-1C was grown in roller bottles as described (1). After the removal of the "sarcoma-conditioned medium," cells were scraped from the bottles into phosphate-buffered saline. After centrifugation at 500 × *g* for 10 min, the supernatant was discarded and the remaining cell pellet was frozen in the gas phase of a liquid nitrogen freezer.

Sarcomas Derived from MuSV-Transformed 3T3 Cells. Cells of the Moloney MuSV-transformed 3T3 cell line 3197-3 (9), deficient in leukemia helper virus, were scraped into sterile phosphate-buffered saline, and 1×10^6 cells were inoculated subcutaneously into *nude* mice. Tumors were harvested at 2 weeks to 2 months and immediately frozen and stored above liquid nitrogen.

Bladder Carcinomas. Highly malignant transplanted carcinomas of passage 4 were kindly provided by R. C. Moon, IIT Research Institute. These tumors were produced by transplantation of a transitional cell carcinoma of the bladder induced in $B_6D_2F_1$ mice by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

Tracheal Carcinomas. Carcinomas originating from injection of a transformed tracheal cell line into heavily immunosuppressed isogeneic recipients were generously provided by Ann C. Marchok, Oak Ridge National Laboratory. The cell line 1000 W was derived from a rat tracheal transplant preexposed to 7,12-dimethylbenz[*a*]anthracene as described by Marchok *et al.* (10).

Soft Agar Assay. The test material was sterilized by lyophilization of 1 M acetic acid solutions in sterile tubes. The residue was then redissolved in binding buffer (11) at 10 times the final concentration used in the assay and centrifuged to clarity. The assay for colony growth in soft agar was modified from that of

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Abbreviations: MuSV, murine sarcoma virus; SGFs, sarcoma growth factors; EGF, epidermal growth factor; TGFs, transforming growth factors.

De Larco and Todaro (1), using normal rat kidney fibroblasts from clone 49F (12). Briefly, samples to be tested were suspended in 0.3% agar (Difco, Noble agar) in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 μg/ml) and containing 3×10^3 cells per ml. A portion (0.7 ml) of the resultant mixture was pipetted onto a 0.7-ml base layer (0.5% agar in the supplemented medium) in 35-mm petri dishes (Falcon Plastics). Plates were then incubated at 37°C for 2 weeks in a humidified 5% CO_2 atmosphere without further feeding. The assay was read unfixed and unstained at 1 week. At 2 weeks, 0.7 ml of a sterile solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (0.5 mg/ml in water) was layered over the agar and the incubation was continued for 24 hr as described by Schaeffer and Friend (13). After removal of excess dye solution, these plates could be scored in a bright-field microscope by projection onto a screen (Fig. 1). The stained plates could be stored for up to 3 weeks at 4°C with no deterioration in readability. Either unstained or stained plates were scored by counting the number of colonies in a 1-cm^2 field. Colonies ranged in size from 0.03 to greater than 0.2 mm. A unit of soft agar colony-forming activity (referred to as "soft agar activity") was defined as the amount of protein that stimulated the formation of one colony (>10 cells) per cm^2 in 2 weeks under the described assay conditions.

Extraction Procedure. Cells or tumors were extracted by a modification of the acid/ethanol procedure of Davoren (3). Tissues (10–130 g) were thawed in 40 ml/10 g of tissue of a solution consisting of 375 ml of 95% (vol/vol) ethanol and 7.5 ml of concentrated HCl, plus 33 mg of phenylmethylsulfonyl fluoride and 1.9 mg of pepstatin as protease inhibitors. The volume was adjusted with distilled water to 60 ml/10 g of tissue and the tissue was minced in a Tissumizer (Tekmar, Cincinnati, OH). After overnight extraction at 4°C, the mixture was centrifuged and the residue was reextracted for 2 hr with 40 ml of a solution consisting of 375 ml of 95% ethanol, 105 ml of distilled water, and 7.5 ml of concentrated HCl. Experimentation showed that although approximately 60–75% of the protein was lost as a precipitate upon adjustment of the pH to 7–8 [part of the insulin purification scheme (3)], over 50% of the soft agar colony-forming activity was lost as well. Therefore, the combined supernatants were kept in the acidic range by adjustment

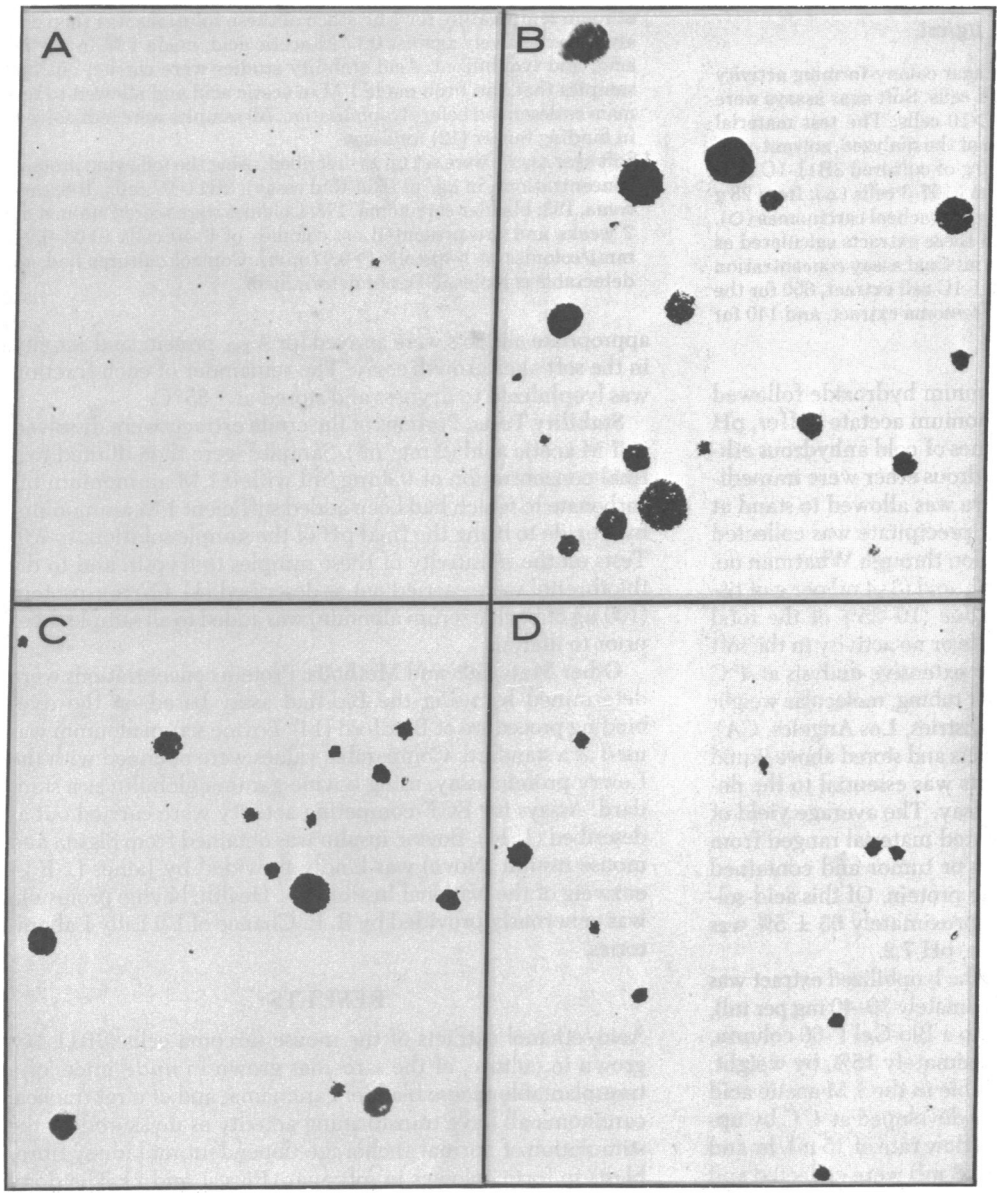

FIG. 1. Soft agar growth of normal rat kidney fibroblasts. Clone 49F cells were plated in 0.3% agar and stained according to Schaeffer and Friend (13). (*A*) Untreated cells; (*B–D*) cells treated with serial 1:3 dilutions (200 μg of protein per ml in *B*) of the acid/ethanol extract of the cultured 3B11-1C cells. Note that both the number of colonies and the size of the colonies decrease with dilution. (×30.)

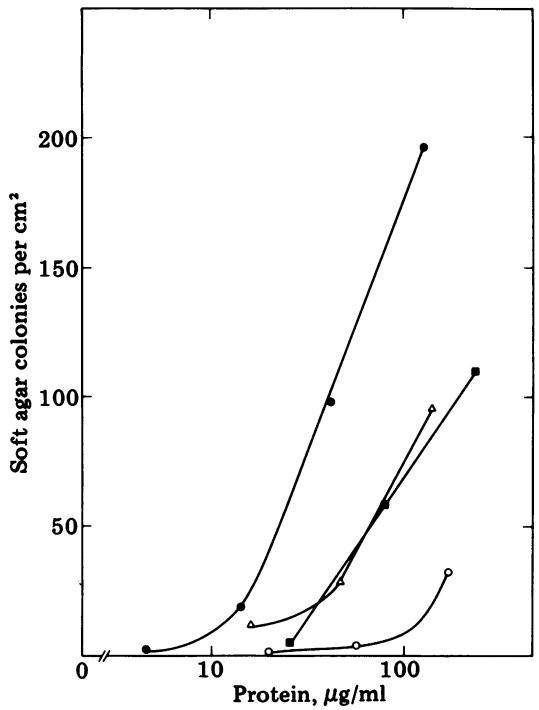

FIG. 2. Dilution curves of the soft agar colony-forming activity of acid/ethanol extracts of transformed cells. Soft agar assays were carried out as described. A colony is >10 cells. The test material represented the pH 7.2 soluble fraction of the dialyzed, solvent-precipitated acid/ethanol extract from 130 g of cultured 3B11-1C cells (●); from 10 g of sarcomas derived from 3197-3 cells (△); from 28 g of bladder carcinomas (■), and from 9 g of tracheal carcinomas (○). The average specific activity of each of these extracts calculated as units of soft agar activity per 1 mg per ml final assay concentration of soluble protein was 1900 for the 3B11-1C cell extract, 650 for the sarcoma extract, 620 for the bladder carcinoma extract, and 140 for the tracheal carcinoma extract.

to pH 5.2 with concentrated ammonium hydroxide followed by the addition of 1 ml of 2 M ammonium acetate buffer, pH 5.3, per 85 ml of extract. Two volumes of cold anhydrous ethanol and four volumes of cold anhydrous ether were immediately added, after which the mixture was allowed to stand at −20°C for 30–48 hr. The resulting precipitate was collected by centrifugation or by rapid filtration through Whatman no. 1 paper and redissolved in 1 M acetic acid (3–4 ml per g of tissue). The acetic acid-insoluble residue (10–25% of the total solvent-precipitated protein) had little or no activity in the soft agar assay and was discarded. After extensive dialysis at 4°C against 0.17 M acetic acid (Spectrapor tubing, molecular weight cutoff 3500, Spectrum Medical Industries, Los Angeles, CA), the samples were lyophilized to dryness and stored above liquid nitrogen. Dialysis of the cell extracts was essential to the detection of activity in the soft agar assay. The average yield of acetic acid-soluble, solvent-precipitated material ranged from 3 to 12 mg/g of original cell pellet or tumor and contained approximately 78 ± 7% acid-soluble protein. Of this acid-soluble protein, an average of only approximately 63 ± 5% was soluble in phosphate-buffered saline, pH 7.2.

Bio-Gel P-60 Chromatography. The lyophilized extract was redissolved in 1 M acetic acid (approximately 30–40 mg per ml), centrifuged to clarity, and applied to a Bio-Gel P-60 column, 100–200 mesh, 2.5 × 70 cm. Approximately 15%, by weight, of the lyophilized extract was insoluble in the 1 M acetic acid and was discarded. The column was developed at 4°C by upward flow of 0.86 M acetic acid at a flow rate of 15 ml/hr and a pressure head of 60 cm. Fractions (3 ml) were collected and

Table 1. Stability tests of the transforming factors as assessed by growth in soft agar

Treatment*	Soft agar colonies per cm² †		
	3B11-1C cells in culture	Sarcoma (MuSV)	Bladder carcinoma
Trypsin control	187/12	177/66	107/4
Trypsin	3/0	1/0	0/0
Dithiothreitol control	212/21	177/32	111/9
Dithiothreitol	2/0	6/1	4/0
Acetic acid control	233/21	139/11	132/5
Acetic acid (30 hr, 4°C)	221/28	119/8	128/10
Acetic acid (30 hr, 20°C)	183/29	155/21	ND
Repeated lyophilization (four times)	242/27	204/30	144/21

* All samples were initially dissolved in 0.1 M NH_4HCO_3. For trypsin treatment, a control solution and one containing trypsin treated with L-tosylamido-2-phenylethyl chloromethyl ketone at 50 μg/ml were incubated at 37°C for 2 hr, at which time soybean trypsin inhibitor was added to give 100 μg/ml. For dithiothreitol treatment, a control solution and one containing 0.065 M dithiothreitol were incubated at room temperature for 1 hr. Each of these solutions was then dialyzed extensively against 0.17 M acetic acid, made 1 M in acetic acid, and lyophilized. Acid stability studies were carried out on samples that had been made 1 M in acetic acid and allowed to remain as described before lyophilization. All samples were redissolved in binding buffer (12) for assay.

† Soft agar assays were set up as described, using the following protein concentrations, in μg/ml (Bio-Rad assay): 3B11-1C cells, 108; sarcoma, 153; bladder carcinoma, 178. Colonies were scored stained at 2 weeks and are presented as: colonies of 6–40 cells (0.03–0.07 mm)/colonies of >40 cells (>0.07 mm). Control cultures had no detectable colonies. ND, not determined.

appropriate aliquots were assayed for A_{280}, protein, and activity in the soft agar growth assay. The remainder of each fraction was lyophilized to dryness and stored at −85°C.

Stability Tests. Portions of the crude extracts were dissolved in 1 M acetic acid (2 mg/ml). Samples were then diluted to a final concentration of 0.4 mg/ml with 0.1 M ammonium bicarbonate to which had been added sufficient 1 M ammonium hydroxide to bring the final pH of the sample solutions to 7.9. Tests on the sensitivity of these samples to trypsin and to dithiothreitol were carried out as described (1). Carrier protein (100 μg of bovine serum albumin) was added to all sample tubes prior to dialysis.

Other Materials and Methods. Protein concentrations were determined by using the Bio-Rad assay based on the dye-binding procedure of Bradford (14). Bovine serum albumin was used as a standard. Comparable values were obtained with the Lowry protein assay, using bovine gamma globulin as a standard. Assays for EGF-competing activity were carried out as described (1, 11). Bovine insulin was obtained from Sigma and mouse insulin (Novo) was kindly provided by James L. Rosenzweig of the National Institutes of Health; bovine proinsulin was generously provided by R. E. Chance of Eli Lilly Laboratories.

RESULTS

Acid/ethanol extracts of the mouse sarcoma cells (3B11-1C) grown in culture, of the sarcomas grown in *nude* mice, of a transplantable mouse bladder carcinoma, and of a rat tracheal carcinoma all have transforming activity as measured by the stimulation of normal anchorage-dependent rat kidney fibroblasts to form colonies in soft agar (Figs. 1 and 2). The dose-

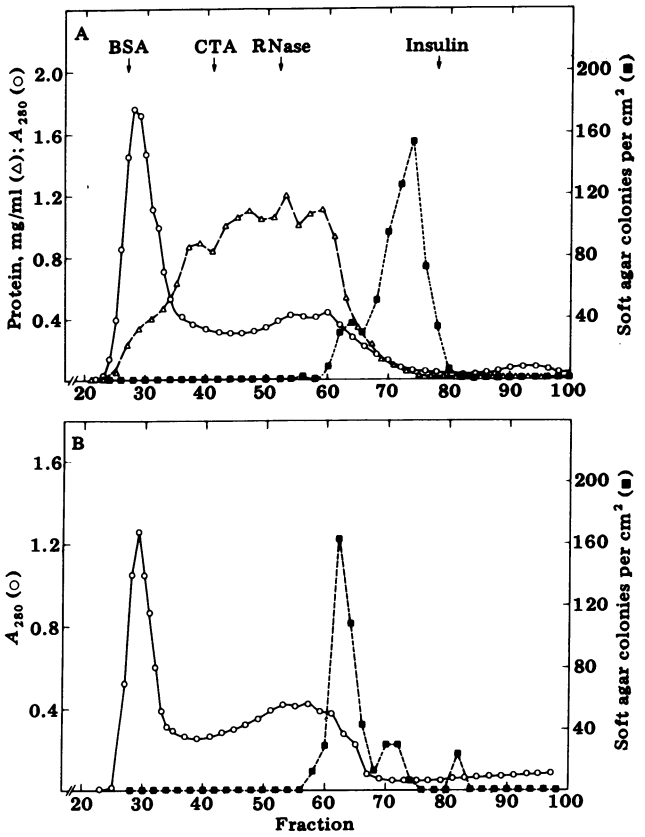

FIG. 3. Bio-Gel P-60 chromatography of the acid/ethanol extract of 3B11-1C cells grown in culture. (*A*) Chromatography of 102 mg of 1 M acetic acid-soluble protein of the dialyzed, lyophilized, solvent-precipitated extract of 130 g of cells. The sample was applied in 6 ml of 1 M acetic acid and eluted as described. Bio-Rad protein analyses were performed on 50-μl aliquots; lyophilized 100-μl aliquots were assayed for soft agar colony formation or for EGF competition. Recovery of protein and of soft agar activity were each 102%. Markers were: BSA, bovine serum albumin (68,000); CTA, chymotrypsinogen A (25,000); RNase (13,800); and insulin (6000). (*B*) Chromatography of 111 mg of the extract of 80 g of another batch of 3B11-1C cells as described above. Fractions having greater than 40% EGF-competing activity in a membrane receptor assay (1, 9) were: in *A*, fractions 69–81 (peak fraction 73), and in *B*, fractions 63–65, 71, and 83 (peak fraction 63).

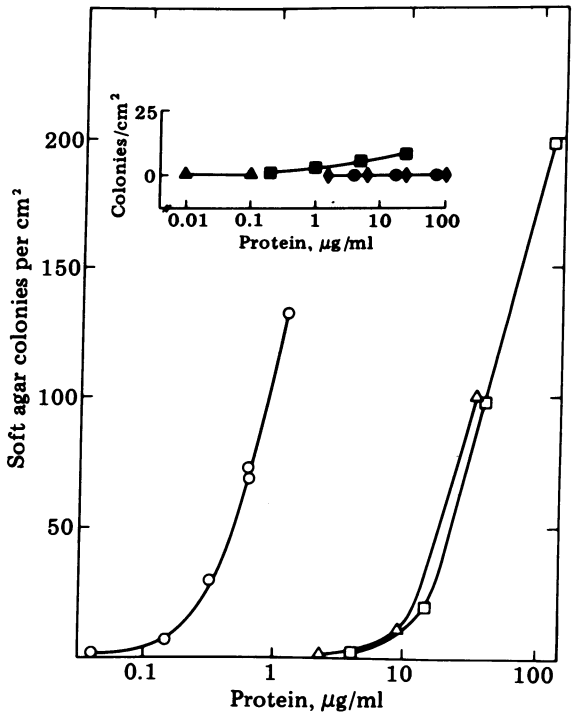

FIG. 4. Dilution curves demonstrating purification of the soft agar colony-forming activity of the acid/ethanol extract of cultured 3B11-1C cells by gel filtration. The test material represented the extract of 3B11-1C cells as described in Fig. 2 (□), or fractions 62 (△) or 74 (○) of the Bio-Gel P-60 chromatography of that extract shown in Fig. 3*A*. The specific activities of fractions 62 and 74 were 2.8×10^3 and 1.1×10^5 soft agar units/mg, respectively. (*Inset*) Test material was mouse insulin (■), bovine insulin (●), bovine proinsulin (♦), and mouse EGF (▲).

response curves shown in Fig. 2 indicate that the extract of 3B11-1C cells has the highest specific activity in the assay, whereas the sarcoma and bladder carcinoma extracts are each about one-third as active. The extract of the tracheal carcinoma has only weak transforming activity, with a specific activity approximately one-tenth that of the cultured cells.

The activity of the TGFs of the 3B11-1C cells, as well as of the sarcoma and bladder carcinoma was destroyed by treatment with trypsin or with dithiothreitol (Table 1), as reported previously for the SGFs of 3B11-1C-conditioned medium (1). The activity of each of these TGFs was stable to 1 M acetic acid for 30 hr either at room temperature or at 4°C, and to repeated lyophilization from 1 M acetic acid solutions.

The acid/ethanol extract of the MuSV-transformed cultured cells was further purified by chromatography at 4°C on Bio-Gel P-60 in 0.87 M acetic acid, as shown in Fig. 3*A*. The soft agar activity was localized in one major peak, in fractions 68–80, with an apparent molecular weight of approximately 7000. This peak contained 2.5% of the total protein recovered from the column and 85% of the soft agar activity of the column (colonies of more than 10 cells). The specific activity of the peak tube (fraction 74) was increased about 50-fold over that of the original extract applied to the column (Fig. 4). Because of the ability of the SGFs from 3B11-1C-conditioned medium to compete with EGF for available membrane receptors (1), odd-numbered column fractions were assayed for EGF competition. Only fractions 69–81 had greater than 40% EGF-competing activity; the peak tube (fraction 73, 93% competition) coincided with the peak tube of soft agar activity. Comparison of the shape of the peak of soft agar activity in tubes 68–80 with that of an insulin standard, which elutes in the same region of the column (fractions 72–90), suggests that the activity of this region could be due to a single protein. In addition, there was a minor peak of soft agar activity localized in fractions 59–66, with an apparent molecular weight of approximately 10,000. This peak contained 17% of the total protein on the column and 11% of the soft agar activity (colonies of more than 10 cells), representing only a small purification (Fig. 4). The soft agar activity of both fractions 62 and 74 was completely destroyed by treatment with either trypsin or dithiothreitol. Recoveries of protein and soft agar activity from the column were routinely 90–110%.

In contrast to the Bio-Gel P-60 profile shown in Fig. 3*A*, which is characteristic of the acid/ethanol extracts of over 200 g of cultured 3B11-1C cells, chromatography of the extract of one preparation of 80 g of these cells resulted in the major peak of soft agar activity (78% of that recovered) at fractions 59–67, with only 18% of the recovered activity appearing in the lower molecular weight region (Fig. 3*B*). As before, the EGF-competing activity coeluted with the soft agar activity. Chromatography of replicate aliquots from each preparation was in every case reproducible. At this time then, it can only be surmised that the observed disparity in the gel chromatography of these extracts results from inherent differences in the par-

ticular cell pellets, and probably not from the extraction procedure or subsequent chromatography.

Due to both the partial overlap of the peak of soft agar activity of the cell extract (Fig. 3A) with the elution profile of a bovine insulin standard and the known efficiency of this extraction procedure for recovering insulin (3, 4), as well as the established similarities between transformed cells and "insulinized" cells (15), the possibility was investigated whether insulin itself could be responsible for colony formation in soft agar. Neither bovine insulin nor bovine proinsulin was able to stimulate normal rat kidney cells to form progressively growing colonies in soft agar (Fig. 4, *Inset*). Mouse insulin at pharmacological concentrations (100 μg/ml) did result in the formation of a few small colonies; this activity, however was insignificant compared to that of fraction 74. Fig. 4 *Inset* also shows that mouse EGF, likewise acid-stable, was inactive in promoting growth of the indicator cells in soft agar, as shown previously (1).

DISCUSSION

It is now apparent from this report that polypeptides capable of stimulating the anchorage-independent growth of normal anchorage-dependent cells are not restricted to the conditioned medium of MuSV-transformed cells growing *in vitro* (1). The intracellular peptides isolated by acid/ethanol extraction of cultured mouse sarcoma (3B11-1C) cells behave identically to the SGFs previously isolated from the conditioned medium of these cells with respect to sensitivity to trypsin and to the disulfide-reducing agent dithiothreitol, the ability to compete for membrane EGF receptors, and the ability to stimulate growth of normal rat kidney fibroblasts in soft agar. The intracellular TGFs of the 3B11-1C cells appear to be comparable in size (Fig. 3) to the components of the extracellular SGFs having apparent molecular weights of 10,000 and 7000 (16). The largest size class of the SGFs (apparent molecular weight 20,000–27,000) is not found in the acid/ethanol extracts of the MuSV-transformed cells.

The finding of transforming polypeptides in acid/ethanol extracts of sarcomas produced by inoculation of MuSV-transformed cells into athymic mice indicates that this property of the cells is not confined to cell culture systems. Furthermore, TGFs are not limited to MuSV-transformed cells, as evidenced by their presence in extracts of chemically induced bladder carcinomas, and to a much lesser extent in extracts of chemically induced tracheal carcinomas. Proof of the chemical and structural relatedness of these peptides must await further purification; however, the stability studies in Table 1 provide evidence that the soft agar activity of each of these TGFs resides in an acid-stable polypeptide containing disulfide bonds necessary for activity. Thus the TGFs are a class of polypeptides common to cells transformed either by chemicals or by sarcoma viruses and possess biological activity distinct from that of the acid-extractable growth factors, insulin and epidermal growth factor.

The ability of these TGFs to enhance colony formation in soft agar is not limited to the use of a clone of normal rat kidney fibroblasts as the indicator cells. It has recently been reported that SGF will stimulate soft agar growth of both an epithelial cell line and a syngeneic fibroblast cell line established from a mouse embryo (17). Unfortunately, given the reversible nature of the phenotypic changes brought about by these transforming peptides (1), it is not feasible to do direct *in vivo* assays for the tumorigenicity of the treated cells. Growth of cells of either fibroblastic or epithelial origin in semisolid medium is, however, acknowledged to be the best *in vitro* correlate of tumorigenicity (18–20) and is therefore used to characterize the biological activity of these peptides.

The difficulties associated with purifying large quantities of material from large volumes of conditioned medium are formidable. This application of an acid/ethanol extraction procedure has been demonstrated to be useful for extracting transforming proteins from samples of 10–130 g of tissue and can easily be scaled up to the processing of kg quantities of starting materials (21). It satisfies the requirements for minimizing proteolytic activity and can extract acid-stable proteins in high yield, as evidenced by the report of recoveries of insulin from the pancreas of greater than 90% with this method (3). Furthermore, there has been much success in purifying to homogeneity and in sequencing proteins with properties that make them amenable to this extraction procedure, among them proinsulin, insulin, glucagon, insulin-like growth factor, and secretin. Thus we propose the acid/ethanol extraction to be a reasonable starting point for the isolation from cells of sufficient TGFs for the eventual determination of their primary structure. Establishment of the amino acid sequence will form the basis for synthesis of agonists and antagonists of these proteins and may lead to a better understanding of the role of this family of peptides in the genesis and maintenance of the transformed phenotype of tumor cells.

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