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Purification and Characterization of a Transformation-Dependent Protein Secreted by Cultured Murine Fibroblasts†

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ABSTRACT: The major excreted protein (MEP) of transformed mouse fibroblasts has been purified, and monospecific antisera against it have been prepared. Synthesis and secretion of this protein have previously been shown to be stimulated by transformation or treatment with tumor-promoting phorbol esters, but its function is still not known [Gottesman, M. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2767–2771; Gottesman, M. M., & Sobel, M. E. (1980) *Cell (Cambridge, Mass.) 19*, 449–455]. The purified protein shows charge heterogeneity by two-dimensional gel electrophoresis; the major intracellular and extracellular species have a molecular weight of 35 000 and a p*I* of 6.8–7.3. The purified secreted protein contains approximately 5–10% neutral sugar by weight and binds specifically to a concanavalin A–Sepharose affinity column. Translation of messenger ribonucleic acid (mRNA) from cells actively synthesizing MEP in cell-free reticulocyte or wheat germ systems, which are reported to be unable to glycosylate translated proteins, results in a product of M_r 33 000 which is presumably devoid of neutral sugar. However, on two-dimensional electrophoresis, the MEP mRNA translation products continue to show charge heterogeneity similar to that seen in intact cells, suggesting that there may be multiple coordinately controlled mRNAs for MEP or a single mRNA species which can be translated in a variety of ways.

Malignant tumor cells are capable of local invasion and distant metastasis and, in many cases, also have effects on their host's blood supply, immune system, nervous system, and ability to assimilate nutrients. Some of these phenomena are presumed to be mediated by biologically active compounds secreted by the tumor cells. Ectopic secretion of a number of bioactive hormones by tumors has been described, and transformed cells in culture have been shown to release a number of factors affecting phenomena such as cell growth (Dulak & Temin, 1973; De Larco & Todaro, 1978), cell migration (Hammond et al., 1974; Burk, 1973), tumor angiogenesis (Klagsbrun et al., 1976), proteolysis (Unkeless et al., 1973; Chen & Buchanan, 1975), and protein phosphorylation (Senger et al., 1979).

Assuming that some of these putative biologically active compounds will be proteins, we have recently investigated the effects of transformation on protein secretion by cultured mouse 3T3 fibroblasts and found that transformation by various RNA viruses, a DNA virus, or chemical carcinogens dramatically stimulates (up to 100-fold) the secretion of a polypeptide of M_r 35 000 which we have called MEP[1] (Gottesman, 1978). Its rate of synthesis and secretion appears to be regulated at a pretranslational level since transformation controls levels of translatable mRNA for MEP (Gottesman & Sobel, 1980). Tumor-promoting phorbol esters also stimulate synthesis and secretion of MEP by regulating translatable mRNA levels (Gottesman & Sobel, 1980). Taken together, these data suggest that MEP may serve as a useful marker of both transformation and tumor promotion and that an

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[1] Abbreviations used: MEP, major excreted protein of transformed mouse fibroblasts; NIH, NIH Swiss 3T3 cells; KNIH, Kirsten virus transformed NIH cells; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; phosphate-buffered saline, Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}.

understanding of the mechanism of its regulation may give insights into these processes. In this work, we describe the purification of, preparation of antibody to, and preliminary characterization of MEP.

Materials and Methods

Cell Lines and Culture Conditions. The NIH Swiss 3T3 nontransformed cell line and its Kirsten virus transformant (KNIH) were originally derived by G. Todaro (National Cancer Institute) and were a gift of C. Scher (Harvard Medical School). Cultures were maintained in Dulbecco–Vogt medium (NIH Media Unit) containing glutamine, penicillin, streptomycin, and 10% calf serum (Colorado Serum Co.) as previously described (Gottesman, 1978). For purposes of MEP purification, cells were grown in glass disposable roller bottles (Wheaton, 750 cm^2). Each roller bottle contained 75 mL of complete medium and was inoculated with 5×10^6 KNIH cells, gassed with CO_2, and incubated at 37 °C in a Bellco roller bottle apparatus until the cells were confluent.

Purification of MEP. KNIH cells were grown in 20 roller bottles until confluent as described above (approximately 2×10^9 cells). Medium was removed by aspiration, and cells in each bottle were washed 3 times by rolling with 20 mL of Dulbecco–Vogt medium lacking calf serum. Cells were then incubated with rolling for 2 h in serum-free medium. The medium was discarded, and the cells were washed for the fifth and final time with serum-free medium as above. Conditioned medium was prepared by rolling each bottle of cells in the presence of 25 mL of serum-free medium for 24 h. The 500 mL of conditioned medium was dialyzed 3 times against 20 L of deionized water and then lyophilized.

The lyophilysate was suspended in 5 mL of 0.1 M Tris-HCl, pH 8.0, and 1 M NaCl and centrifuged at 100000*g* for 60 min, and the supernatant was concentrated to a volume of 1.0–1.5 mL by pervaporation before application to a Sephadex G-75 column (0.9 × 50 cm). The column was calibrated with Blue Dextran, bovine serum albumin (BSA), cytochrome *c*, and vitamin B-12 as molecular weight markers. MEP, assayed by gel electrophoresis (see below), eluted as a symmetrical peak of ultraviolet (UV) absorbing material between the elution positions of BSA and cytochrome *c*. The MEP peak was pooled, dialyzed against 0.01 M Tris, pH 8.0, applied to a DEAE-52 cellulose column, and eluted with a linear salt gradient (0–0.5 M NaCl). The MEP eluted at an ionic strength of 0.1 M.

A summary of the overall purification of MEP showing one of five different preparations appears in Table I and the Sephadex and DEAE chromatograms are shown in Figures 1 and 2. In one preparation used for metal analysis, care was taken to avoid adventitious contamination by heavy metals by using metal-free buffers and acid-washed glassware as previously described (Drum et al., 1969). A variation on this purification procedure in which the conditioned medium was diluted 5-fold with water and concentrated by batch adsorption to DEAE gave reduced overall yields of MEP, but significantly shortened the preparation time.

Preparation of Antibody to MEP. MEP (100 μg) was mixed with Freund's incomplete adjuvant and inoculated subcutaneously in multiple sites in each of two rabbits. Inoculations were given every 2 weeks. After four such injections, both rabbits were found to have high titers of anti-MEP antibody. Double immunodiffusion analysis using Hyland Ouchterlony plates was as previously described (Gottesman, 1978).

Radiolabeling of Cultured Cells. Cultured NIH or KNIH cells were metabolically labeled in culture with mixed ^{14}C-labeled amino acids (New England Nuclear), and secreted proteins were Cl_3AcOH precipitated as previously described (Gottesman, 1978). In some experiments, cells were labeled with [^{35}S]methionine (New England Nuclear or Amersham, approximately 1000 Ci/mmol) in Dulbecco–Vogt medium lacking methionine (NIH Media Unit). In one experiment, cells were labeled for 3 h with 0.5 mCi carrier-free $^{33}P_i$ in Dulbecco–Vogt medium lacking phosphate.

Immunoprecipitations. Immunoprecipitations of medium, whole cell lysates, and in vitro translation mixtures were performed by a modification of the *Staphylococcus aureus* method of Kessler (1975). Cell lysates were prepared by treatment of cells with buffer A (0.154 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.5% Nonidet P-40, and 0.05% $NaDodSO_4$) and clarified by centrifugation at 12000*g* for 5 min. The sample containing MEP was diluted into 1 mL of buffer A in a 1.5-mL Eppendorf tube and incubated for 30 min at 4 °C with 5 μL of preimmune or anti-MEP rabbit serum which had been heated for 10 min at 56 °C and clarified by centrifugation at 12000*g* for 5 min. Formalinized *Staph. aureus* (Bethesda Research Laboratories) (50 μL of a 10:1 suspension of a *Staph. aureus* pellet in buffer A) was added, and, after an additional 30 min at 4 °C, the samples were centrifuged for 90 s at 12000*g* in an Eppendorf microcentrifuge (Model 5412). The *Staph. aureus* pellets were washed by resuspension and recentrifugation with 0.8 mL of each of the following solutions: 3 times with buffer B (buffer A lacking $NaDodSO_4$ and containing 2.5 M KCl), once with buffer A, and once with deionized water. The final *Staph. aureus* pellets were treated with $NaDodSO_4$ dissociation buffer (see below) before electrophoresis. Evidence for quantitative immunoprecipitation was obtained as previously described (Gottesman & Sobel, 1980).

Direct immunoprecipitates were obtained by addition of 10 μL of antiserum to 1 mL of cell lysate (1% Triton X-100 in phosphate-buffered saline) containing radiolabeled MEP and 1–2 μg of unlabeled MEP. Precipitates were allowed to form for 18 h at 4 °C and were collected by centrifugation at 15000*g* for 30 min.

One- and Two-Dimensional Gel Electrophoresis and Fluorography. One-dimensional gels of samples dissolved in $NaDodSO_4$ dissociation buffer (2.5% $NaDodSO_4$, 10% glycerol, 5% 2-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8) were run as described by Laemmli (1970) except that the separating gel contained 10% glycerol (Cabral & Schatz, 1979). Immunoprecipitates and radiolabeled cells for two-dimensional gel analysis were also dissolved in $NaDodSO_4$ dissociation buffer and then acetone precipitated as previously described (Cabral & Gottesman, 1978). Medium for two-dimensional gel analysis was Cl_3AcOH precipitated (Gottesman, 1978). The protein pellet was redissolved in 8 M urea–5% Triton X-100–1% 2-mercaptoethanol and run on isoelectric focusing gels containing 1.2% pH 3.5–10 Ampholines (LKB) followed by $NaDodSO_4$ gel electrophoresis in 10% polyacrylamide gels containing 10% glycerol (Cabral & Schatz, 1979). Stained gels were prepared for fluorography after destaining as described by Bonner & Laskey (1974).

Carbohydrate, Metal, and Amino Acid Analyses. Carbohydrate analysis was performed by the phenol–sulfuric acid method as described by Ashwell (1966). Excess BSA (140 μg) was added to all of the reaction tubes as a non-carbohydrate-containing protein blank, and the calibration curve was determined with glucose as the standard. Ovalbumin (Sigma) was used as a glycoprotein control and gave a color reaction similar to that of MEP when the same amount of Lowry

Table I: Purification of MEP Secreted by KNIH Cells

step	volume (mL)	protein concn[a] (mg/mL)	total protein (mg)	MEP (fraction)[b]	yield (%)
2×10^9 KNIH cells + medium	500.0	~2.0	~1000	0.015	
conditioned medium	500.0	~0.1	~50	0.3	100
solubilized, dialyzed lyophilysate	5.1	8.5	43.4	0.3	86
concentrated 10^5g supernatant	1.35	22.2	30.0	0.3	67
Sephadex G-75, pooled peak fractions	11.0	0.77	8.9	1.0	60
DEAE, pooled peak fractions	3.0	1.0	3.0	1.0	20

[a] Protein concentration was measured according to Lowry et al. (1951). [b] The fraction of total protein which was MEP was estimated from Coomassie Blue staining of polyacrylamide gels, except in steps 1 and 2 where the estimates are based on metabolic labeling data.

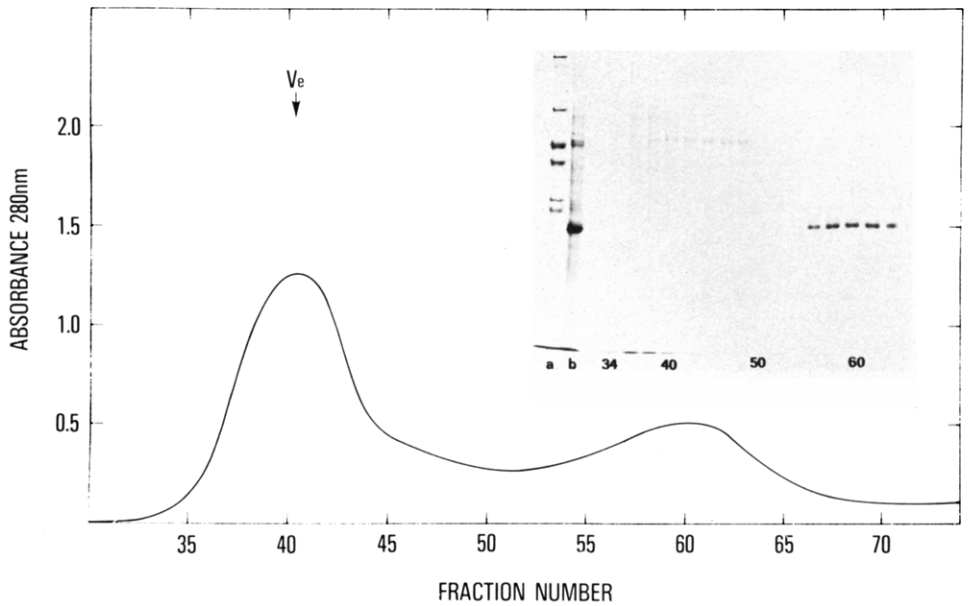

FIGURE 1: Sephadex G-75 chromatography of conditioned medium from KNIH cells. The medium was prepared and applied to the column as described under Materials and Methods. The inset shows the Coomassie Blue staining patterns of polyacrylamide gels of the starting material and representative fractions. (a) Molecular weight standards; (b) starting material.

protein was added. Metal analysis was by microwave excitation helium plasma emission spectrometry as previously described (Kawaguchi & Vallee, 1975). Amino acid analyses were performed on 24-, 48-, and 72-h hydrolysates and 24-h performic acid oxidized samples with a Beckman amino acid analyzer.

Concanavalin A–Sepharose Affinity Chromatography. Concanavalin A–Sepharose was obtained from Pharmacia. A 1-mL column containing 10 mg of concanavalin A was washed with 20 mL of buffer containing 25 mM Hepes, pH 7.5, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Concentrated conditioned medium from KNIH cells (~500 μg of protein) which had been dialyzed against this starting buffer was applied to the column. The column was washed with 5 mL of starting buffer, 5 mL of starting buffer containing 0.2 M galactose (Sigma), and 5 mL of starting buffer containing 0.2 M methyl α-mannoside (Sigma), and 1-mL fractions were collected and analyzed for protein by Coomassie Blue staining of polyacrylamide gels.

Neuraminidase and Alkaline Phosphatase Treatment. Conditioned medium from KNIH cells (1 mL) in which secreted proteins had been labeled with mixed ^{14}C-labeled amino acids was adjusted to pH 7 with CO_2 and treated with 10 μg of neuraminidase from *Clostridium perfringens* (Sigma, Type VI) for 30 min at 37 °C or with 90 μg of alkaline phosphatase from *Escherichia coli* (Sigma) at pH 8 for 20 min at 37 °C. The reactions were terminated by addition of 20 μg of BSA and 0.1 mL of 100% Cl_3AcOH (w/v). Cl_3AcOH pellets were analyzed by two-dimensional gel electrophoresis.

Tryptic Peptide Maps and in Vitro Translations. Two-dimensional tryptic peptide maps of protein spots excised from two-dimensional gels were as described previously (Shih et al., 1978). Purification of RNA from KNIH cells by a modification of the guanidine extraction method (Cox, 1967; Adams et al., 1977; Strohman et al., 1977) and its translation in vitro have been described (Sobel et al., 1978; Gottesman & Sobel, 1980).

Results

Purification of MEP. MEP was purified from conditioned serum-free medium obtained from KNIH cultures and was assayed as a Coomassie Blue staining band of M_r = 35 000 seen on one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis. As shown in Table I and detailed under Materials and Methods, the secretion step itself constitutes a 30-fold purification of MEP from total cell proteins plus contaminating serum proteins (mostly bovine serum albumin). By gel filtration on Sephadex G-75, it was possible to obtain a symmetrical peak of protein which chromatographed with M_r = 30 000–40 000 and which was essentially free of contamination by other Coomassie Blue staining material (Figure 1). The MEP was applied to a DEAE-cellulose column and eluted at 0.1 M NaCl to separate it from nucleic acids which eluted at high ionic strengths (Figure 2). Overall purification was 100-fold from the original culture and approximately 3–4-fold from the conditioned medium (Table I). The amino acid analysis of the purified protein is shown in Table II.

Preparation and Specificity of Antibody to MEP. Antisera were prepared in each of two rabbits as described under Materials and Methods. After four inoculations, antisera from

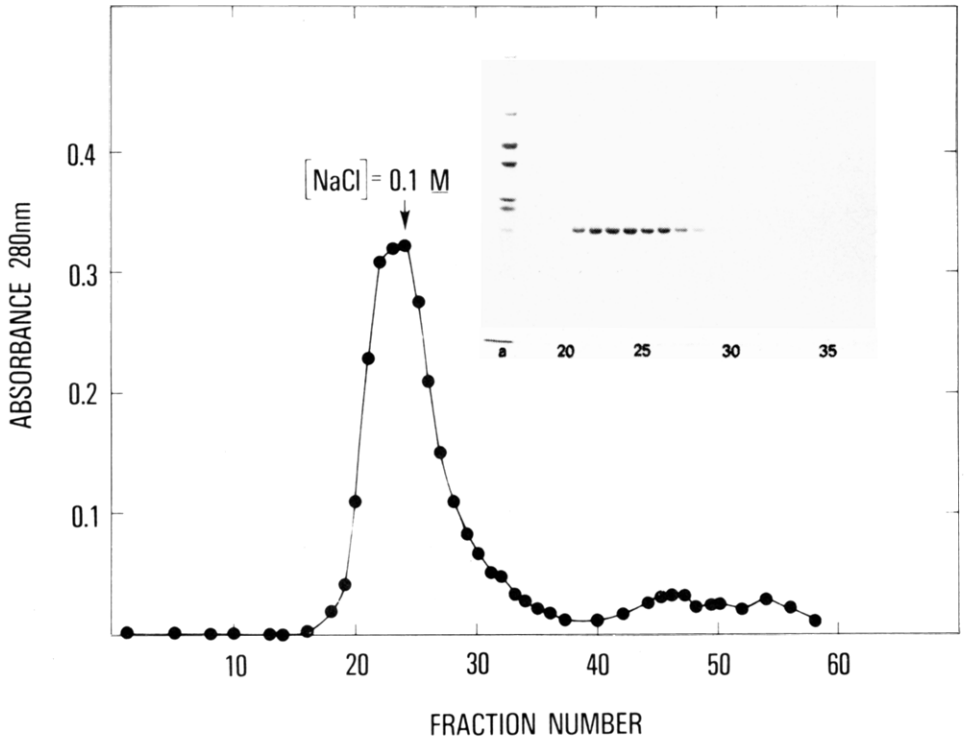

FIGURE 2: DEAE-52 chromatography of MEP previously purified by gel filtration. Details appear under Materials and Methods. The inset shows the Coomassie Blue staining patterns of polyacrylamide gels of representative fractions. (a) Molecular weight standards.

Table II: Amino Acid Analysis of MEP[a]

Asp	31.7	Met	12.8
Thr	11.1	Ile	13.3
Ser	22.0	Leu	25.1
Glu	44.5	Tyr	20.8
Pro	12.3	Phe	19.0
Gly	32.2	His	9.0
Ala	19.2	Lys	23.0
Val	12.5	Arg	11.2
		Cys (oxidized)	10.2

[a] Average of 24- and 48-h hydrolyses; number of residues assuming 320 total amino acid residues.

both rabbits gave single, strong precipitin lines against purified MEP on Ouchterlony gels, and neither reacted with any serum proteins, including purified bovine serum albumin (data not shown). The specificity of one of the antisera was determined more completely by immunoprecipitation of labeled extracts, and this antiserum was used in all subsequent experiments. Figure 3 shows the monospecific immunoprecipitation of MEP (Figure 3C) from a [^{35}S]Met-labeled detergent lysate of KNIH cells (Figure 3A). The preimmune serum from the same rabbit did not react with any KNIH proteins (Figure 3B). The MEP immunoprecipitated from the KNIH cells comigrated with unlabeled Coomassie Blue stained MEP purified from KNIH conditioned medium and included in the immunoprecipitation reaction. Judged by precipitation of this unlabeled MEP, precipitation was quantitative. Similar results could be obtained by using direct precipitation without *Staph. aureus* (Figure 3D–G), but the results were not always quantitative; hence, this technique was not used in subsequent work. MEP has been specifically immunoprecipitated from pulse-labeled cell lysates of a large number of transformed mouse and rat cell lines with no evidence to date of cross-reaction with any other normal cellular proteins (data not shown).

Heterogeneity of MEP. When we ran two-dimensional gels of purified MEP, it became obvious that the material we had purified from conditioned medium was heterogeneous with

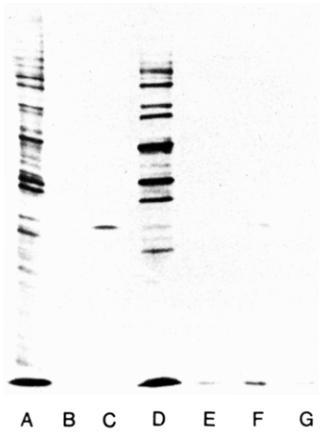

FIGURE 3: Specificity of anti-MEP rabbit serum. KNIH cells were pulse-labeled for 30 min with [^{35}S]Met (A–C) or with ^{14}C-labeled amino acids (D–G) and detergent lysates prepared as described under Materials and Methods. Immunoprecipitation was performed by use of *Staph. aureus* (A–C) or by direct addition of antiserum (D–G). (A) KNIH lysate (20 000 cpm); (B) control precipitation with preimmune serum (200 000 cpm); (C); precipitation with anti-MEP serum (200 000 cpm); (D) KNIH lysate (10 000 cpm); (E) control precipitation with preimmune serum (100 000 cpm); (F) precipitation with anti-MEP serum (100 000 cpm); (G) control precipitation with immune serum from a rabbit inoculated with tubulin but not containing any precipitating antibody to this protein. The arrow shows the position of MEP.

respect to both molecular weight and charge. In subsequent experiments, it became obvious that the molecular weight heterogeneity was largely due to inadequate boiling in our $NaDodSO_4$ dissociation buffer, and we have been able to alleviate this problem by boiling for at least 5 min in dissociation buffer containing 5% 2-mercaptoethanol. For determination of whether the charge heterogeneity might be due to degradation or modification during the long periods in which the medium was in contact with the cultured cells prior to purification, medium containing MEP pulse-labeled with

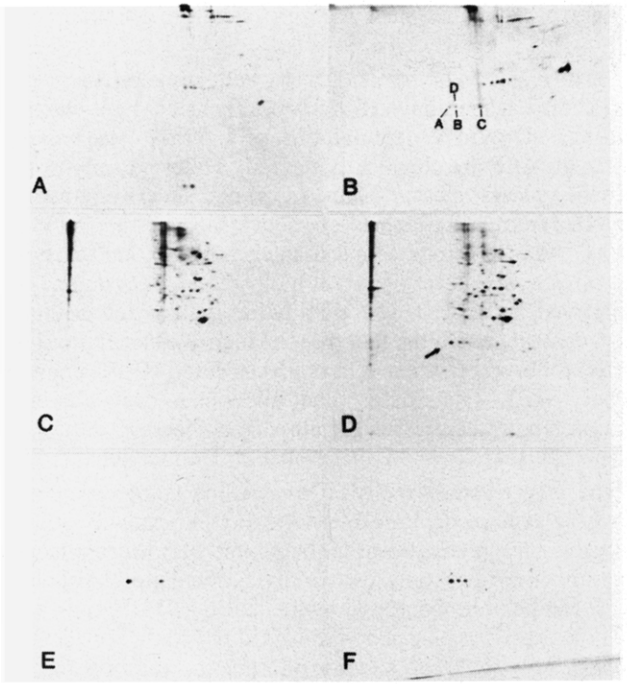

FIGURE 4: Two-dimensional gel analysis of cell lysates, conditioned medium, and immunoprecipitates. Cells were labeled for 30 min with [^{35}S]Met (C, D) or were labeled for 30 min after which medium was collected after 3 h of secretion (A, B), and gels were run as described under Materials and Methods. (A) Conditioned medium from NIH cells (nontransformed); (B) medium from KNIH (transformed) cells. The letters denote MEP spots excised from another [^{35}S]Met-labeled gel and analyzed for methionine-containing tryptic peptides (Figure 5); (C) whole cell lysate from NIH cells; (D) lysate from KNIH cells (the arrow points to the cluster of MEP spots); (E) immunoprecipitate from KNIH conditioned medium; (F) immunoprecipitate from KNIH whole cell lysate. The horizontal dimension is isoelectric focusing with the acidic proteins to the right, and the vertical dimension is $NaDodSO_4$ gel electrophoresis.

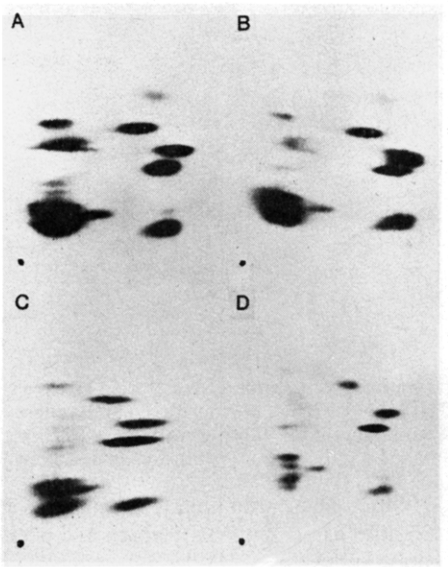

FIGURE 5: Two-dimensional tryptic peptide analysis of [^{35}S]Met-containing peptides from MEP. The spots indicated in Figure 4B were cut from the gel and tryptic peptic maps prepared as described under Materials and Methods. Some minor differences in the maps may be due to technical problems with the resolution of peptides.

[^{35}S]Met and collected soon after secretion of the labeled proteins was analyzed. This analysis revealed heterogeneity similar to that seen with the purified, unlabeled protein (Figure 4). In this experiment, MEP can be identified either as the labeled material which is secreted by the transformed KNIH cells (Figure 4B) but not by the nontranformed cells (Figure 4A), preferentially synthesized in pulse-labeled transformed cells (Figure 4D) as compared to nontransformed cells (Figure 4C), or by immunoprecipitation out of medium using antisera prepared against secreted MEP (Figure 4E). Figure 4F shows that MEP immunoprecipitated out of [^{35}S]Met-labeled whole cell lysates is similarly heterogeneous; hence, the heterogeneity cannot be due to processing in the medium. Also, as seen in Figure 4B, the p*I* of the major species is approximately 7.0, but spots are seen over a pH range of 6.8–7.3. Calibration of these gels by carbamoylation (Cabral & Gottesman, 1978) indicates that the spots differ by more than unit charges.

For confirmation that the clustered low molecular weight transformation dependent protein spots were all related proteins, the [^{35}S]Met-labeled spots were excised from the two-dimensional gels and subjected to two-dimensional tryptic peptide analysis. Figure 5 compares the methionine-containing tryptic peptides of several of the excised spots identified in Figure 4B. It is apparent that they all have very similar, if not identical, tryptic fingerprints.

Glycosylation of MEP. Analysis of purified MEP for neutral sugars indicated approximately the same ratio of sugar to protein as was seen in an ovalbumin control known to contain 5–10% neutral sugar (w/w) (data not shown). As seen

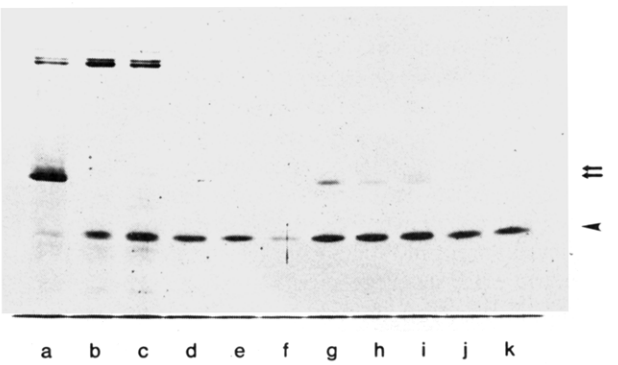

FIGURE 6: Concanavalin A–Sepharose chromatography of KNIH conditioned medium. Chromatography was as described under Materials and Methods. Coomassie Blue staining patterns of 10% polyacrylamide gels are shown: (a) conditioned KNIH medium; (b–d) buffer wash; (e, f) 0.2 M galactose wash; (g–k) 0.2 M α-methyl mannoside wash. The arrows mark the portion of MEP, and the arrowheads show concanavalin A which elutes continuously from this column.

in Figure 6, MEP from conditioned medium binds to a concanavalin A–Sepharose affinity column, and is specifically eluted by methyl α-mannoside. These data taken together indicate that MEP is a glycoprotein.

The proteins in conditioned medium were digested with neuraminidase to remove terminal sialic acid residues to test whether charge heterogeneity of MEP was due to variable sialylation. This treatment did not alter the pattern of MEP spots, although it did affect patterns of several other secreted proteins in a manner consistent with desialylation (data not shown).

Translation of MEP in a Cell-Free System. In order to determine the contribution of pre- and posttranslational components to MEP heterogeneity, we isolated total RNA from KNIH cells and translated it in either rabbit reticulocyte or wheat germ systems in the presence of [^{35}S]Met. In both cases, antiserum to MEP specifically precipitated two protein bands as displayed on one-dimensional $NaDodSO_4$ gels (Gottesman

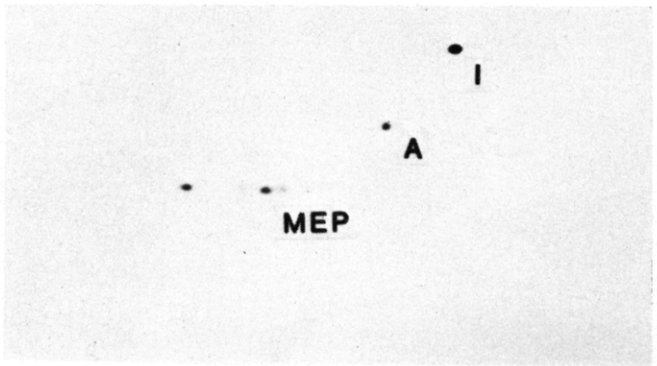

FIGURE 7: Immunoprecipitation of MEP from in vitro translations of total KNIH RNA. RNA was purified, and immunoprecipitates of in vitro translations of this RNA were prepared as described under Materials and Methods. Spots are labeled as described in the text.

& Sobel, 1980). The predominant band, with M_r 33 000, migrated slightly faster than MEP which had been immunoprecipitated out of lysates of labeled intact KNIH cells. The in vitro translated MEP had a tryptic peptide map indistinguishable from MEP labeled in intact cells (Gottemsan & Sobel, 1980). The altered mobility of in vitro synthesized MEP was thought to be due to lack of glycosylation. The second, minor band of M_r 45 000 was precipitated only out of the in vitro translation reaction mixtures and not from lysates of intact cells. It had a different tryptic peptide map from MEP. In addition, a third band of M_r 58 000 was precipitated by both preimmune and immune sera and in subsequent experiments was shown to precipitate with *Staph. aureus* alone.

Figure 7 shows a two-dimensional gel of the immunoprecipitate from a reticulocyte lysate in vitro translation of total KNIH RNA. The in vitro translated protein still shows considerable charge heterogeneity, with a pattern similar to that seen for MEP synthesized in intact cells (Figure 4). The simplest interpretation of these data is that glycosylation, which is believed absent in reticulocyte lysates, does not cause the isoelectric MEP heterogeneity.

The spot labeled A represents the other in vitro translated protein (M_r 45 000) specifically immunoprecipitated by antiserum against MEP (M_r 35 000). The spot labeled I, seen in both the preimune and immune precipitations, is probably fibroblast intermediate filament protein because it migrates in this gel system in the same position as intermediate filament protein purified from Chinese hamster ovary fibroblasts (Cabral & Gottesman, 1979; Cabral et al., 1980). Intermediate filament protein is a sparingly soluble protein which either nonspecifically precipitates out during the treatment of extracts with *Staph. aureus*, or actually binds to the formalinized bacteria.

Discussion

The major excreted protein (MEP) of transformed mouse fibroblasts has been purified by a simple two-step procedure, and specific antisera have been prepared against the purified protein. These antisera have already proved valuable in the quantitation of MEP synthesized in nontransformed cells after treatment with tumor-promoting phorbol esters (Gottesman & Sobel, 1980) and in the visualization of MEP secretion granules in intact transformed cells by indirect immunofluorescence (Gottesman, 1978). Because MEP synthesis seems to be regulated both by transformation (Gottesman, 1978) and tumor promoters (Gottesman & Sobel, 1980), an understanding of the factors controlling its synthesis could give insights into these biologically important processes. The antisera described in this work will make it feasible to determine MEP synthesis rates under a variety of culture conditions and in a number of different cell types.

Purified MEP shows heterogeneity both in molecular weight and charge when analyzed by two-dimensional gel electrophoresis. Tryptic peptide mapping proves that these various forms of MEP are chemically related. Heterogeneity in the protein appears to occur prior to secretion since immunoprecipitates from pulse-labeled extracts also show this heterogeneity. MEP precipitated out of in vitro translation reactions migrates with a lower apparent molecular weight on one-dimensional $NaDodSO_4$ gels than MEP synthesized in intact cells, consistent with the fact that the higher molecular weight form produced by intact cells is glycosylated (Gottesman & Sobel, 1980). These data do not allow us to draw any conclusions about the presence or absence of "leader" sequences in in vitro translated MEP (Blobel & Dobberstein, 1975).

The charge heterogeneity of MEP which we observe is still not fully explained. Variable sialylation is probably not responsible since pretreatment of the sample with neuraminidase does not alter the pattern on two-dimensional gels. Although MEP can be phosphorylated by incubating KNIH cells with $^{33}P_i$, the degree of phosphorylation is slight (data not shown). Treatment with *E. coli* alkaline phosphatase removes the ^{33}P but does not affect the charge heterogeneity of the bulk of the protein (data not shown), implying that phosphorylation does not cause the charge heterogeneity. We analyzed one of our preparations of MEP for heavy metals and found ≤ 0.2 mol of Zn, Cu, Mn, or Fe per mol of protein, consistent with minor trace metal contamination. Since metal–protein complexes should not be stable to the denaturation conditions used in our gels (8 M urea), we feel that these metals are also unlikely to be responsible for the charge heterogeneity of MEP.

The amino acid analysis (Table II) shows a large excess of acidic over basic residues, but the p*I* of our major species is 6.8, suggesting the possibility that variable posttranslational amidation or deamidation of asparagine or glutamine residues might be contributing to some extent to the charge heterogeneity of MEP. Other modifications, such as acetylation or methylation, could also account for this charge heterogeneity. We find a similar heterogeneity of MEP after in vitro translation of purified RNA (Figure 7). This implies either that the rabbit reticulocyte lysate has the same modifying activities as the intact mouse KNIH cell, that there is actual heterogeneity of the mRNA species which code for MEP, or that a single mRNA can be translated in a number of ways. If the multiple mRNA hypothesis is correct, then the stimulation of translatable mRNA for MEP by transformation represents an example of a coordinately regulated system in a eukaryotic cell.

We have been unable to assign a specific biological role to MEP. Tests for protease activity with benzoylarginine ethyl ester or labeled proteins secreted by NIH cells as substrates, activity as a protease inhibitor, growth or migration-promoting activity, and tumor angiogenesis activity with the purified protein have so far been negative. Medium from KNIH cells does contain plasminogen activator activity, but this activity does not comigrate with MEP on $NaDodSO_4$–polyacrylamide gels (R. O. Roblin and M. M. Gottesman, unpublished results). MEP synthesis is not only limited to 3T3 fibroblasts; MEP can be detected in a variety of transformed mouse and rat cell lines (Gottesman, 1978, and unpublished data) and is increased in primary mouse epidermal cells after treatment with tumor promoters (M. M. Gottesman, F. Cabral, and S. H. Yuspa, unpublished results). Recently, other laboratories have reported the secretion of novel polypeptides by cultured cells.

Hynes and his co-workers have detected a polypeptide of M_r 60000 in the culture media of transformed hamster cells which appears to have protein kinase activity (Senger et al., 1979). Nilsen-Hamilton and co-workers (1980) have described a family of low molecular weight polypeptides secreted by Swiss 3T3 cells under the influence of polypeptide mitogens which they call mitogen-released proteins (MRPs). A subset of these MRPs includes MEP. As for MEP, the biological function of any of these proteins remains as yet undefined.

Even in the absence of a knwon function, however, studies of MEP synthesis, glycosylation, and secretion made possible by the chemical characterization reported in this work should provide important information concerning both transformation and tumor promotion.

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