Purified Scatter Factor Stimulates Epithelial and Vascular Endothelial Cell Migration (43115)

ELIOT M. ROSEN, LEONID MEROMSKY, EVA SETTER, DAVID W. VINTER, AND ITZHAK D. GOLDBERG

Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut 06510; Department of Radiation Therapy, Harvard Medical School, Boston, Massachusetts 02115; and Department of Radiation Oncology, Long Island Jewish Medical Center, New Hyde Park, New York 11042

Abstract. **Fibroblasts and smooth muscle cells release a protein activity which causes epithelial sheets to "scatter" into isolated cells. Purification of scatter factor (SF) activity from *ras*-transformed 3T3 cells was reported recently. We purified ras-3T3 SF by a slightly different method with essentially similar findings. Purified factor showed a single band at 77 ± 3 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. Scatter activity was eluted from gel slices at this molecular size. Reduction with mercaptoethanol caused the loss of activity and the appearance of two bands (58 and 31 kDa). We report the amino acid composition of ras-3T3 SF and sequences of several tryptic peptides. These sequences were not similar to the known proteins in the Protein Database. We have shown previously that partially purified *ras*-3T3 scatter activity stimulates migration of epithelial and vascular endothelial cells in a new migration assay utilizing microcarrier beads. We now demonstrate that the same purified ras-3T3 protein scatters epithelial cells and stimulates epithelial and endothelial migration in microcarrier bead and Boyden chamber assays. Partially purified human smooth muscle scatter activity shares these activities, but the protein(s) responsible has not been isolated. Migration-stimulating activity was maximal at ras-3T3 protein concentrations <10 ng /ml (0.13 n*M*). ras-3T3 SF had no collagenolytic activity and did not stimulate DNA synthesis in fibroblast growth factor-responsive human melanocytes. ras-3T3 SF appears to be a new protein which regulates endothelial and epithelial mobility; and, therfore, it may be involved in vascular repair and wound healing.**

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Several proteins appear to be specific regulators of movement in nonleukocytic cells. Yoshida *et al.* (1) described a 70-kDa protein produced by metastatic tumor cells which acts as a chemoattractant for the producer cells. A2058 human melanoma and other metastatic tumor cells release a 54-kDa autocrine motility factor (AMF) which stimulates pseudopod protrusion and migration of the producer cells (2, 3). A similar 53-kDa factor was obtained from rat mammary carcinoma cells (4). A 70-kDa migration-stimulating factor (MSF) released by fetal and cancer patient fibroblasts stimulates penetration of nonproducing fibroblasts into three-dimensional collagen gels (5, 6). A protein activity which "scatters" contiguous sheets of epithelium was found in culture medium from human embryonic fibroblasts (7), variants of 3T3 and their transformed derivatives (8), and bovine and human arterial smooth muscle cells (9). We reported that partially purified fibroblast-derived scatter activity stimulates migration of both epithelial and vascular endothelial cell types in a new quantitative assay utilizing microcarrier beads (10). Gherardi *et al.* (11) recently reported purification of scatter factor (SF) from a high producer line of *ras* oncogene-transformed mouse 3T3 fibroblasts. We now describe our technique for rapid purification of large quantities of fibroblast-derived SF, confirm the findings of Gherardi, and report further characterization studies, including amino acid analysis and partial amino acid sequencing. We show that the purified scatter protein is responsible for both epithelial and vascular endothelial migration-stimulating activities in microcarrier bead and Boyden chamber assays.

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Materials and Methods

Sources of Cell Strains and Lines. *SF producer cells.* ras-3T3 (D4). ras-3T3 (D4) is a high producer cloned line of *ras* oncogene-transformed NIH/2 3T3 mouse fibroblasts (8). Cells were provided by Dr. Mi-

chael Stoker (Department of Pathology, Cambridge University, Cambridge, UK).

Vascular smooth muscle cells. Adult human iliac artery smooth muscle cell strains (HIAS 112,113) derived from explants of the arterial media were provided by Dr. Elliot Levine (Wistar Institute, Philadelphia, PA) at about population doubling 20. Calf aortic smooth muscle cells (ASM2) were isolated from media explants and studied from passage 8–12 (9).

SF target cells.. Madin-Darby canine kidney (MDCK). MDCK epithelial cells were obtained from the American Type Culture Collection (CCL34) (Rockville, MD).

Vascular endothelial cells. Calf pulmonary artery endothelial cells (CPAE) were obtained from the American Type Culture Collection (CCL209) at passage 16 and were studied from passage 18–22. Calf aortic endothelial cells isolated by us (EC-30, EC-31) were studied from passage 10–20. Human umbilical vein endothelial cells (HUVE) were provided by Dr. William Carley (Department of Anesthesia, Yale University School of Medicine, New Haven, CT) at passage 7. All four strains showed characteristic cobblestone endothelial monolayer morphology with very few "sprouting" cells and immunofluorescent staining for Factor VIII antigen (12).

Nonproducer fibroblasts. BALB/c 3T3 is a nontransformed, non-SF-producing line of mouse fibroblasts obtained from the American Type Culture Collection (CCL163).

Cell Culture Techniques. Stock cultures of cell strains and lines were grown in 100-mm plastic petri dishes (Corning 25020, Corning, NY) or 150-mm dishes (Falcon 3025; Becton-Dickinson, Oxnard, CA) in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 0.1 m*M* nonessential amino acids, 5.0 mg/ml D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and fetal bovine serum (medium, nonessential amino acids, antibiotics, and serum were obtained from Grand Island Biological Co., Grand Island, NY). Serum concentrations were 5% for ras-3T3 cells; 10% for ASM2, MDCK, EC-30/31, and BALB/c 3T3; and 20% for HIAS, CPAE, HUVE. HIAS and HUVE growth media were also supplemented with crude endothelial cell growth factor from bovine hypothalamus and heparin (provided by Dr. Levine), as described previously (13). Cultures were harvested weekly (every 4–5 days for ras-3T3) using trypsin (0.25% trypsin, 0.1% EDTA, calcium-magnesium-free; Hazelton Research Products, Lenexa, KS). Stock dishes were inoculated at split ratios of 1:20 (ras-3T3), 1:10 (ASM2, MDCK, EC-30/31, CPAE, BALB/c 3T3), and 1:4 (HIAS, HUVE) in growth medium (15 ml/100-mm dish, 30 ml/150-mm dish) and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. HIAS 112/113, MDCK, CPAE, HUVE, and BALB/c 3T3 were tested initially and found to be mycoplasma-free by the suppliers.

Purification of ras-3T3 SF. *Collection of conditioned medium (CM).* ras-3T3 cells were seeded into 1750-cm^2 plastic roller bottles (Falcon 3035) (three confluent 150-mm dishes/roller) and incubated in DMEM, 5% serum (300 ml/roller) for 4–5 days at 0.8 rpm. Confluent rollers were washed three times with serum-free DMEM to remove serum proteins and incubated with 175 ml of serum-free DMEM for 48 hr. CM was collected, centrifuged to remove debris (3000 rpm × 20 min), and stored at −20°C for up to 2 months until used for chromatography.

Chromatography. Crude ras-3T3 CM was purified at 22°C using: Bio-Rex 70, a weak cation exchanger (no. 142-5842; Bio-Rad, Richmond, CA); S-Sepharose fast flow, a strong cation exchanger (no. 17-0547-01; Pharmacia); and VYDAC C4 reverse-phase high-performance liquid chromatography (HPLC). Details are provided in the legend to Figure 2. Purity of fractions was monitored by specific scatter activity (scatter units per μg of protein). Scatter activity was quantitated by MDCK dilution assay (see below); protein concentration was measured using the Bio-Rad assay (14) or estimated from UV absorbance at 280 nm.

Partial Purification of HIAS SF. *Collection of CM.* HIAS cells were grown to confluency in 150-mm petri dishes for 1–2 weeks with refeeding twice weekly. Culture dishes were washed three to four times with serum-free DMEM and incubated for 48 hr with serum-free DMEM (15 ml/dish). CM was centrifuged (3000 rpm × 20 min) and stored at −20°C.

Chromatography. CM was partially purified 200-fold by cation-exchange chromatography using S-Sepharose fast flow, as described previously (15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Vertical SDS-discontinuous polyacrylamide gel electrophoresis was performed as described by Laemmli (16). Two parts sample were mixed with one part 3 × sample buffer to a final buffer composition of 62.5 m*M* Tris (pH 6.8), 2% SDS, 10% glycerol, ±5% 2-mercaptoethanol. The sample was boiled for 2–3 min and electrophoresed on a 1-mm-thick 5–20% acrylamide gradient gel using a Bio-Rad model 500/200 power supply (30–60 mA, approximately 5 hr). Gels were silver stained, and molecular weight values were estimated from plots of log (MW) versus R_F of standard proteins. For gel elution experiments, samples were not boiled or reduced (no mercaptoethanol), the separating gel was 12.5% acrylamide, and the preparative portion of the gel was not stained.

SDS-substrate gel (gelatin zymogram). Gelatin zymogram analysis was performed by Dr. William Stetler-Stevenson (Laboratory of Pathology, National Cancer Institute), as described previously (17). The separating gel was 9% acrylamide copolymerized with 0.1%

gelatin. Samples were diluted in sample buffer containing 0.1% SDS and electrophoresed without heating or reduction. The gel was washed in 2.5% Triton X-100, incubated overnight in 0.05 *M* Tris-HCl, 0.02 *M* NaCl, 0.005 *M* $CaCl_2$, pH 7.6 at 37°C, and stained in 0.1% Coomassie blue. Zones of clearing correspond to gelatinolytic activity.

Bioassays of SF Activity. *MDCK scatter assay.* Scatter activity (i.e., SF concentration) was quantitated by a dilution assay in 96-well microtiter plates (Falcon 3072), as described previously (7, 9). Briefly, 5000 MDCK cells/well were incubated at 37°C in serial dilutions of sample in 300 μl of DMEM containing 5% serum. Cells were stained after 20 hr incubation and observed for scattering (spreading or normally tight cohesive colonies and separation into isolated cells (Fig. 1B)). The highest dilution at which scattering was present was defined as having 0.5 MDCK scatter units/ml, allowing calculation of SF concentration (units/ml) and specific activity (units/μg protein). Assays were reproducible to within ±0.5 dilution (factor of 2). Scatter titers and effects were similar whether assays were performed using freshly trypsinized MDCK cells or preattached (1- or 2-day-old) colonies.

Boyden chamber assay. Assays were performed in 48-well microwell chambers from Neuroprobe (Cabin John, MD) using collagen-coated Nucleopore polycarbonate filters (18, 19) containing 8-μm pores. To assay endothelial and 3T3 cells, SF was diluted in DMEM containing 2.5 mg/ml bovine serum albumin (DMEM-BSA); to assay MDCK cells, factor was diluted in DMEM containing 1% fetal calf serum (DMEM-1%). Dilutions of SF (30 μl) were placed in triplicate in the lower wells. Cells from confluent stock cultures were detached with trypsin, resuspended, centrifuged twice (800 rpm × 5 min) to remove trypsin, counted using a hemacytometer, and resuspended at 2×10^6 cells/ml. Fifty microliters of a suspension in DMEM-BSA or DMEM-1% (as above) was placed in each upper well. Chambers were incubated at 37°C for 4 hr (endothelial, 3T3 cells) or 18 hr (MDCK). Filters were then fixed and stained using Diff-Quick Chemicals (American Scientific Products, McGaw Park, IL). Nonmigrated cells on the upper side of the filter were removed with a cotton swab, and nuclei of migrated cells on the underside of the filter were coutned using a × 40 microscope objective. Results were expressed as migrated cells per $mm^2 \pm 1$ SEM ($n = 3$).

Microcarrier bead migration assays. We described a new migration assay based on movement of target cells off microcarrier beads onto plastic culture surfaces (10). Briefly, cells were cultured for 2–3 days on Cytodex 2 microcarrier beads (Pharmacia no. 17-0484-01), and then cells were counted (20). Beads were seeded into 2-cm^2 wells in 24-well plates at 1×10^5 cells in 0.5 ml of DMEM-5% serum/well. Dilutions of SF were added in triplicate in a small volume (<20 μl), and cultures were incubated at 37°C for 6 hr or 18 hr (depending on intrinsic migration rates of target cells). Beads were removed by rinsing with buffered saline, and cells attached to the plastic were stained with crystal violet and counted using a ×10 objective. Migration was expressed as cells per 10 fields (approximately 0.2 cm^2) ± 1 SEM ($n = 3$).

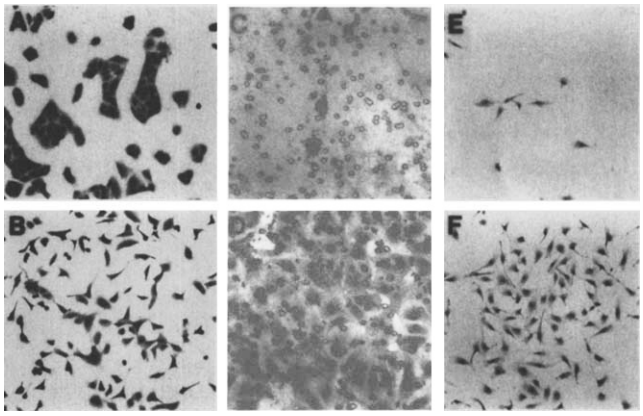

Figure 1. Epithelial cell scattering and stimulation of vascular endothelial cell migration by ras-3T3-derived scatter factor. (A and B) Epithelial scatter assay. Five thousand MDCK cells were incubated in the absence (A) or presence (B) of scatter factor (4 units/ml) for 20 hr at 37°C. Cultures were stained with crystal violet and photographed (original magnification × 160). (C and D) Endothelial Boyden chamber assay. Migration of calf pulmonary artery endothelial cells in the absence (C) or presence (D) of factor (20 units/ml) in the lower well (original magnification × 160). (E and F) Endothelial microcarrier bead assay. Migration of bovine aortic endothelial cells (strain EC-30) from microcarrier beads onto plastic culture wells in the absence (E) or presence (F) of factor (40 units/ml) (original magnification × 160).

Amino Acid Analysis and Sequencing. Ion-exchange amino acid analysis of purified ras-3T3 SF was performed at the Yale Protein and Nucleic Acid Chemistry Facility, using a Beckman model 7300 Amino Acid Analyzer. Sequencing was carried out at the Yale Protein and Nucleic Acid Chemistry Facility using an Applied Biosystems model 477 Sequencer with on-line phenylthiohydantoin detection (Applied Biosystems model 120A). Direct analysis of 50 pmol of ras-3T3 SF did not yield a sequence. Two-hundred picomoles of acetone-precipitated protein was reduced, carboxamidomethylated, and digested with trypsin (1:50 t/t) for 24 hr. Tryptic peptides were isolated on a 4.6 mm × 25 cm VYDAC C18 HPLC column and repurified on an Aquapore C8 column. Sequences were searched against the Protein Sequence Database (National Biomedical Research Foundation, Washington, DC). The Mutation Data Matrix was used to evaluate homology with known proteins.

Photomicroscopy. Photomicrographs were made using a Zeiss IM35 microscope with green filter, ×10 objective, and Olympus OM4T 35-mm camera attachment. Magnification of the negative was ×80. The film was Kodak Plus-X (ASA 125) or T-Max 100.

Results

Purification of ras-3T3 SF. We purified scatter activity about 10,000-fold from ras-3T3 serum-free CM by cation-exchange plus reverse-phase chromatography (Fig. 2). Crude CM contained 0.5–1.0 MDCK scatter units/μg of protein. Sequential chromatography using Bio-Rex 70, S-Sepharose (two passes), and C4 HPLC yielded active fractions with specific activities of 50–100, 500–1,000, and 5,000–10,000 units/μg. Elution from S-Sepharose and C4 columns required high concentrations of salt (0.7 *M* NaCl) and organic solvent (80% acetonitrile), respectively, suggesting that factor contains basic and hydrophobic domains. For rapid purification of large volumes of CM, the HPLC step may be omitted; factor can be purified by low-pressure cation-exchange chromatography and preparative electrophoresis (see below).

Purified ras-3T3 SF showed a single band at 77 ± 3 kDa on nonreduced silver-stained SDS-polyacrylamide gels (Fig. 3). To verify the identity of this band, gel elution experiments using partially purified SF were

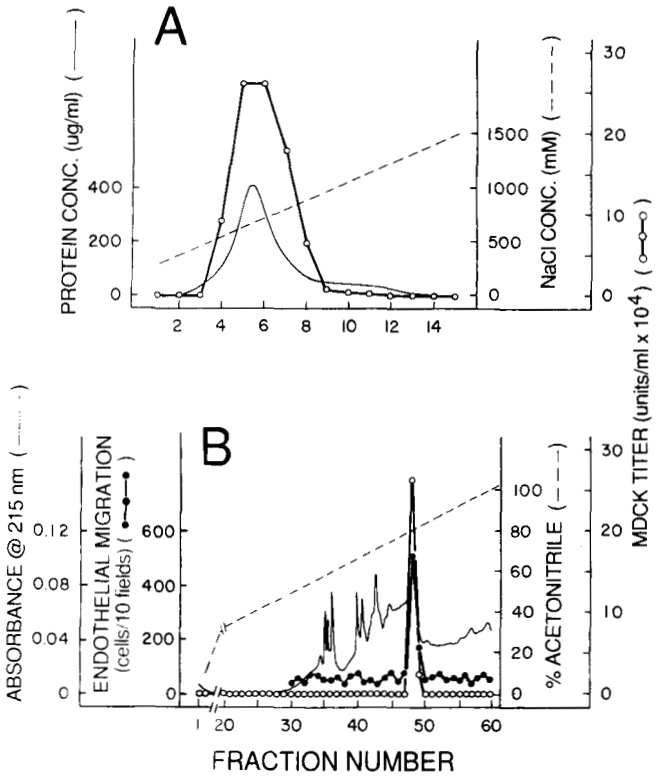

Figure 2. Purification of ras-3T3 scatter activity. (A) last step in cation-exchange chromatography. Five liters of ras-3T3 serum-free CM were passed over a 250-ml bed Bio-Rex 70 column (6 × 9 cm) at about 500 ml/hr. The column was washed with 1000 ml of 0.3 *M* NaCl in Tris buffer, 10 m*M* (pH 7.5) and eluted with 600 ml of 1.0 *M* NaCl in the same buffer. The eluant was diluted to 0.25 *M* NaCl and loaded on a 25-ml bed S-Sepharose column (2.5 × 5 cm) at 150 ml/hr. This column was washed with 100 ml of 0.3 *M* NaCl and eluted with 75 ml of 1.0 *M* NaCl. The eluant was then diluted to 0.25 *M* NaCl and loaded on a 1.0-ml bed S-Sepharose column (0.8 × 2 cm) at 60 ml/hr. The latter was eluted with a 15 ml of 0.3–1.5 *M* NaCl gradient (in 10 m*M* Tris, pH 7.5), and 1.0-ml fractions were collected and assayed for scatter activity (A). About 65% of the activity in CM was recovered, with about 1000-fold purification. (B) C4 reverse-phase HPLC. Five-tenths milliliters of S-Sepharose fraction 6 (1.3×10^5 units, 150 μg of protein) was made to 1.0 g/liter trifluoroacetic acid and loaded on a 2.4-ml bed VYDAC C4 column. The column was eluted at 1.0 ml/min with a 30-min gradient (0–100%) of acetonitrile in water (containing 1.0 g/liter of trifluoroacetic acid). Fractions (0.5 ml) were lyophilized, redissolved in 0.1 ml of buffered saline, and assayed for MDCK scatter activity and for stimulation of endothelial cell migration from microcarrier beads. (Dilutions (1:5000) of fractions were incubated with calf pulmonary artery endothelial cells for 6 hr and migration was measured as described in Materials and Methods.) Recovery of activity was about 20%.

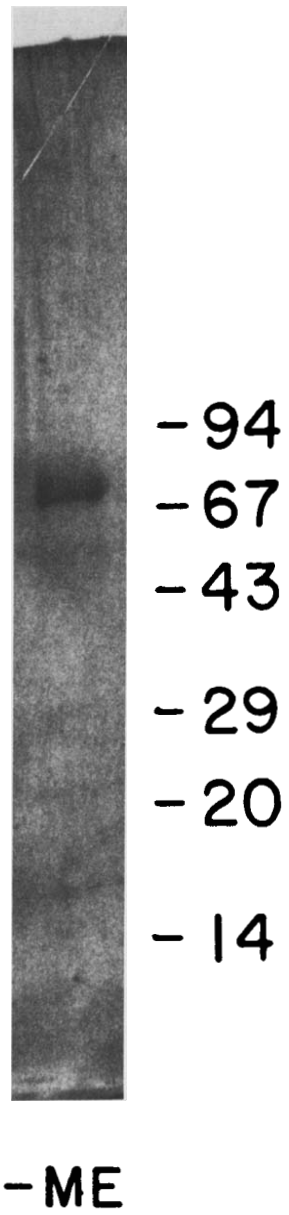

Figure 3. SDS-polyacrylamide gel electrophoresis of ras-3T3 SF under nonreducing conditions. An aliquot (10 μl) of highly purified ras-3T3 SF (Fraction 48 from C4 HPLC run in Fig. 2B) was analyzed on a silver-stained 5–20% gradient gel (16), with mercaptoethanol (ME) omitted from the sample buffer. Lines show positions of reduced molecular mass standards: 94 kDa (phosphorylase b), 67 kDa (bovine serum albumin), 45 kDa (ovalbumin), 29 kDa (carbonic anhydrase), 20 kDa (soybean trypsin inhibitor), 14 kDa (lysozyme).

performed (Fig. 4). In five experiments, activity was eluted from unstained gel slices corresponding to this band (about 5–35% of the loaded activity was recovered). Reduction of chromatographically purified ras-3T3 SF with mercaptoethanol resulted in appearance of two bands (58 and 31 kDa) on SDS-PAGE (Figs. 4 and 5). In some cases, a 90-kDa band was also present (Fig. 5A). Reduction of SF eluted at 75–80 kDa from nonreduced gel slices revealed the same 58- and 31-kDa bands plus a faint 90-kDa band (Fig. 5B). Thus, the 58-, 31-, and 90-kDa species may all originate from the same protein, which migrates at 77 kDa in the nonreduced state.

One explanation for these findings is that ras-3T3 SF is a heterodimer containing 58- and 31-kDa disulfide-linked subunits. The difference in observed (77 kDa) and expected (89 kDa) molecular size on nonreduced gels could reflect intra- plus interchain disulfide

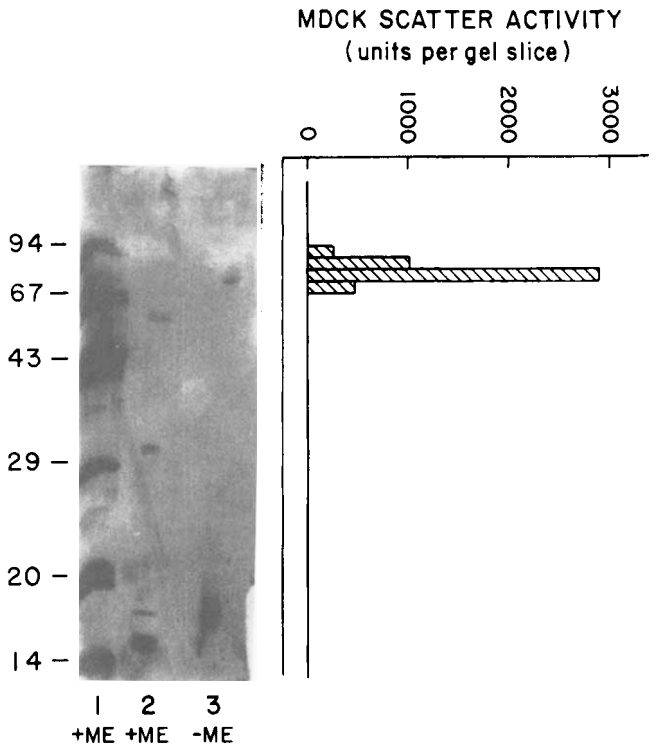

Figure 4. Elution of scatter activity from SDS-polyacrylamide gel slices. The preparative portion of a 12.5% SDS-polyacrylamide gel was loaded with ras-3T3 SF partially purified by cation-exchange chromatography (0.9 ml, 20,880 units), using a 4-cm slot. Factor was not reduced or boiled, to preserve activity. The reference portion of the gel was loaded with molecular weight markers and with ras-3T3 SF from the same chromatographic fraction. The gel was electrophoresed and the reference portion was silver stained, whereas the preparative part was sliced into $n = 40$ three-mm slices. Slices were eluted by diffusion for 24 hr at 22°C in 1.0 ml of 10 m*M* Tris buffer (pH 7.5) containing 0.15 *M* NaCl; eluates were assayed for MDCK scatter activity. About 22% of the total loaded activity was recovered from gel slices corresponding to about 75–80 kDa. Lane 1, reduced molecular weight standards (see Fig. 3 legend); Lane 2, reduced sample (50 μl, 1160 units); Lane 3, nonreduced sample (50 μl, 1160 units). ME, mercaptoethanol.

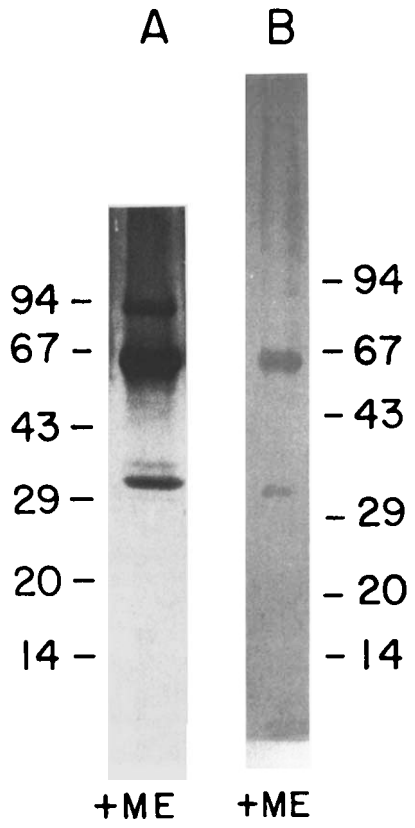

Figure 5. SDS-polyacrylamide gel electrophoresis of ras-3T3 SF under reducing conditions. (A) An aliquot (25 μl) of highly purified factor (Fraction 48 from C4 run in Fig. 2B) was reduced with 5% mercaptoethanol (ME) in sample buffer and analyzed on a silver-stained 5–20% gradient gel. (B) Factor eluted from gel slices at 75–80 kDa (Fig. 4) was dialyzed against distilled water for 24 hr and concentrated to 100 μl using Sephadex beads. An aliquot (30 μl) was reduced and analyzed on a silver-stained 5–20% gradient gel.

bonding, allowing a compact configuration for the complex. The 90-kDa species could represent partially reduced protein with disrupted intrachain but intact interchain –SS– bonds. Alternatively, factor may be a monomer which is cleaved proteolytically at a specific site but held together by disulfide bonds. Reduction releases the 31-kDa fragment. The 90-kDa band could represent noncleaved protein.

Properties of ras-3T3 SF. Properties of ras-3T3-derived SF are summarized in Table I. Factor was sensitive to heat and to trypsin, as was previously reported for fibroblast and smooth muscle-derived scatter activities (7, 9). Heating at 65°C for 30 min, incubation with 10 μg/ml trypsin at 37°C for 30 min, or incubation with 100 μg/ml trypsin for 5 min reduced activity by 90% or more. Activity was stable under alkaline conditions (pH 9 or 10), was reduced at acid pH, and was destroyed by reductive alkylation.

Near-maximal scattering of MDCK epithelial cells (Fig. 1B) occurred at 4 units/ml, corresponding to ras-3T3 protein concentrations of 0.4–0.8 ng/ml (0.005–0.01 n*M*, assuming molecular mass of 77 kDa). Endo-

Table I. Properties of ras-3T3 Scatter Factor

Treatment	MDCK scatter activity remaining[a] (%)
50°C × 30 min	79
55°C × 30 min	41
60°C × 30 min	28
65°C × 30 min	6
70°C × 30 min	5
100°C × 5 min	2
Incubation with trypsin[b]	
1 μg/ml × 30 min	100
10 μg/ml × 30 min	9
100 μg/ml × 30 min	2
100 μg/ml × 1 min	78
100 μg/ml × 5 min	8
100 μg/ml × 15 min	2
pH[c]	
3	25
4	25
5	60
6	86
8	86
9	86
10	100
Reductive alkylation[d]	<5

[a] ras-3T3 SF used in these studies was purified partially by cation exchange chromatography (Fig. 2A). Values are averages from two to three experiments, with each assay condition tested in duplicate.
[b] SF was incubated with trypsin (Type I, T8003, Sigma) in serum-free DMEM at 37°C. Trypsin was stopped with soybean trypsin inhibitor (SBTI) (Sigma T9777) (200 μg/ml). For control, SBTI was added before trypsin.
[c] Aliquots of factor were adjusted to indicated pH with HCl or NaOH, incubated for 90 min at 22°C, neutralized to pH 7.2, adjusted to the same final volume with buffer, and assayed.
[d] SF was incubated with dithiothreitol or mercaptoethanol (10 m*M*) for 90 min at 37°C to reduce disulfide bonds. Reduced SF was incubated with iodoacetamide (20 m*M*) for 60 min at 37°C to prevent reoxidation. Factor was dialyzed against distilled water for 48 hr at 4°C. Controls were incubated for (90 + 60) min without reducing agent or iodoacetamide and were similarly dialyzed.

thelial migration-stimulating activity (discussed below) copurified with MDCK scatter activity on cation-exchange chromatography and reverse-phase HPLC (Fig. 2B). Migration-stimulating activity was present in fractions which were electrophoretically homogeneous on nonreduced SDS-polyacrylamide gels, suggesting that epithelial scatter and vascular endothelial migration-stimulating activities are due to the same protein. Stimulation of endothelial cell migration in Boyden chamber and microcarrier bead assays is illustrated in Fig. 1D and F, respectively. Maximal responses occurred at about 40 scatter units/ml, corresponding to 4–8 ng/ml (0.05–0.10 n*M*).

Secretion of metalloproteinases (e.g., collagenases, stromelysin) may enhance invasiveness of tumor cells (21) and migration of endothelial cells during angiogenesis (22). To determine if ras-3T3 SF has proteolytic activity, SDS-substrate gel (gelatin zymogram) analysis was performed (Fig. 6). This assay localizes proteases by molecular weight as zones of clearing (17). ras-3T3 SF (70 ng) had no gelatinolytic activity whereas type IV collagenase (1.5 ng) caused marked clearing. This finding is consistent with our previous observation that various antiproteases did not block scatter activity (9).

Amino Acid Analysis and Sequence. Table II compares the amino acid composition of ras-3T3 SF, autocrine motility factor (2), and migration-stimulating factor (6). Differences are apparent in the three factors. For example, MSF has no methionine, whereas SF and AMF contain methionine; SF contains less tyrosine and arginine and more histidine than AMF. Direct sequencing of 50 pmol of ras-3T3 SF did not yield a sequence, suggesting that the peptide subunits are aminoterminally blocked. 200 pmol of SF was digested with trypsin, and four tryptic peptides were isolated and sequenced. Examples of continuous, unambiguous sequences are: (i) Val-Gly-Tyr-Glu-Ser-Glu-Ile-Pro-Lys and (ii) Val-Thr-Leu-Asn-Glu-Ser-Glu-Leu-Cys-Ala-Gly-Ala-Glu-Lys. Two additional sequences contained several unknown residues: (iii) Asn-Pro-Asp-Gly-Ala-Glu-Ser-Pro-*X*-*X*-Phe- and (iv) *X*-Glu-Glu-Gly-Gly-Pro-Lys-*X*-Phe-Thr-Ser′-Asn-*X*-*X*-Val- (Ser′ = serine or cysteine). None of these peptides matched or showed significant homology with sequences in the Protein Database. (Maximal homology scores for sequences 1 and 2 were 31 of 45 and 39 of 58, respectively, using the Mutation Data Matrix.) Thus, ras-3T3 SF appears to be a new protein.

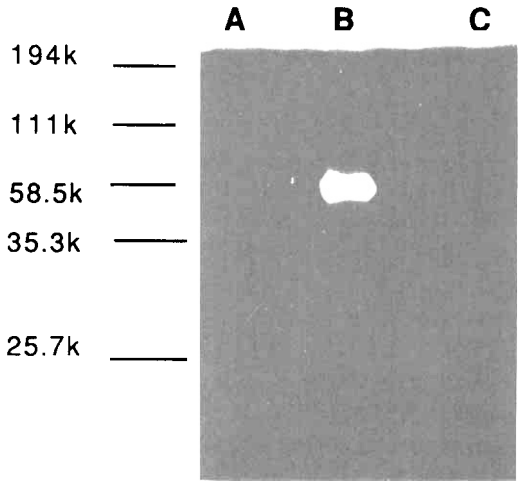

Figure 6. SDS-substrate gel (gelatin zymogram) analysis of ras-3T3 SF. Assay was performed as described previously (17). Lane A contained prestained molecular weight markers. Lane B was a positive control consisting of 15 μl of A2058 human melanoma cell conditioned medium, containing about 1.5 ng of Type IV collagenase. Lane C had 15 μl of partially purified ras-3T3 SF (estimated to contain about 70 ng of SF). Molecular weight markers were reduced with dithiothreitol, whereas samples were run unreduced to preserve enzyme activity. The sensitivity of the assay is about 0.05–0.1 ng of purified Type IV collagenase.

Table II. Amino Acid Composition of Purified ras-3T3 Scatter Factor[a] Compared with Other Cell Mobility Factors

Amino acids	Residues/100		
	ras-3T3 SF	AMF	MSF
Asx	8.1	7.5	7.5
Thr	4.6	4.0	5.2
Ser	17.4	12.0	11.4
Glx	14.0	12.5	9.1
Pro	3.6	4.5	13.6
Gly	23.0	22.9	17.2
Ala	6.4	6.5	5.3
Val	3.9	3.8	4.1
Met	0.6	2.2	—
Ile	2.6	1.8	2.6
Leu	3.9	4.9	6.3
Tyr	2.2	4.1	1.2
Phe	1.9	2.1	2.9
His	3.2	2.0	4.6
Lys	2.6	3.4	5.7
Arg	2.2	5.7	3.3

[a] Data are shown -Trp-Cys. Analysis of ras-3T3 SF was performed twice. The average deviation for each amino acid was 10%. AMF and MSF data were obtained from Refs. 2 and 6, respectively.

Stimulation of Vascular Endothelial Cell Mobility. The production of scatter activity by arterial smooth muscle cells (9) suggested that SF might act on an arterial target: the endothelial cell. Endothelial cells cannot be studied in scatter assays because they do not usually grow as tight cohesive colonies. We used two quantitative assays to demonstrate stimulation of endothelial cell migration by purified ras-3T3 SF and by partially purified human iliac artery smooth muscle (HIAS) factor (Fig. 7). SF concentration was expressed as MDCK scatter units per ml.

Migration across collagen-coated filters was measured with SF in the lower wells of microwell Boyden chambers. Calf pulmonary artery endothelial cell (CPAE) migration was stimulated about 20-fold in the presence of 40 units/ml purified ras-3T3 factor. A similar response was obtained using HIAS-derived factor partially purified 200-fold by S-Sepharose chromatography (Fig. 7B). Migration of human umbilical vein endothelial cells was stimulated 3-fold by both ras-3T3 and HIAS factors, with dose-response curves similar to CPAE (Fig. 7B). Both factors also markedly stimulated migration of MDCK epithelial cells, with 50-fold maximal responses at 20–30 units/ml (Fig. 7A). Endothelial and epithelial migration rates were increased whether factor was in the lower well only or in both upper and lower wells (data not shown), suggesting that SF stimulates random motility of its target cells. SF had little or no effect on migration of producer ras-3T3 cells (Fig. 7C) or nonproducer BALB/c 3T3 cells (Fig. 7D).

Purified ras-3T3 SF and partially purified HIAS SF markedly stimulated migration of endothelial cells off microcarrier beads onto plastic culture surfaces. Maximal migration rates for CPAE (Fig. 7F) and for several strains of bovine aortic endothelium were 4–10 times control values. Migration of MDCK was stimulated about 4-fold (Fig. 7E). Endothelial and epithelial cells showed similar dose-response relationships, with maximal migration at 30–50 scatter units/ml. ras-3T3 and BALB/c 3T3 cells did not respond to factor (Figs. 7G and 7H).

ras-3T3 SF was assayed for fibroblast growth factor (FGF)-like bioactivity in melanocyte DNA synthesis assays (Table III). DNA synthesis in cultured human melanocytes is markedly stimulated by basic FGF (2 ng/ml) in the presence of cyclic AMP (23). ras-3T3 SF at 10 and 100 units/ml (1.3 and 13 ng/ml, using the estimate of 7500 units/μg for pure protein) did not affect melanocyte DNA synthesis. We reported that basic FGF, acidic FGF ± heparin, and endothelial cell growth factor did not affect endothelial migration in bead assays (10). Thus, it is unlikely that endothelial migration stimulation was due to contamination of ras-3T3 SF with FGF.

Effect of SF on Cell Proliferation. We reported that smooth muscle-derived SF did not affect epithelial proliferation at high (5%) or low (1%) serum concentration (9). In additional studies, subconfluent cultures of bovine aortic endothelial cells (EC-30), MDCK cells, and bovine aortic smooth muscle cells (ASM-2) were exposed to different concentrations of ras-3T3 SF (1–500 units/ml) in DMEM-0.2% serum, and cells were counted after 4 days. Factor had little or no effect on cell number, whereas all three cell types responded to additional serum (data not shown). Thus, SF was not mitogenic under the conditions studied.

Discussion

We purified scatter activity from CM from *ras*-transformed mouse 3T3 cells using low-pressure cation-exchange chromatography (Bio-Rex 70, S-Sepharose) plus C4 reverse-phase HPLC. Low-pressure chromatography allows rapid purification of large volumes of CM, to the point where SF is a major band (10–20% of the total protein). Such preparations can be purified further to electrophoretic homogeneity by preparative electrophoresis (Figs. 4 and 5B). Purified chromatographic fractions showed a single 77-kDa band on nonreduced SDS-polyacrylamide gels. Activity was eluted from gel slices corresponding to this band. Gherardi *et al.* (11) purified ras-3T3 SF from CM concentrated with an Amicon P30 membrane, using ion-exchange HPLC (Mono-S and Mono-Q), and C8 reverse-phase HPLC. They found a single 62-kDa band on nonreduced gels. The difference in nonreduced molecular weight in the present study and that of Gherardi could have resulted from technical factors in the gel preparation or from differential cleavage of sugar side chains during the

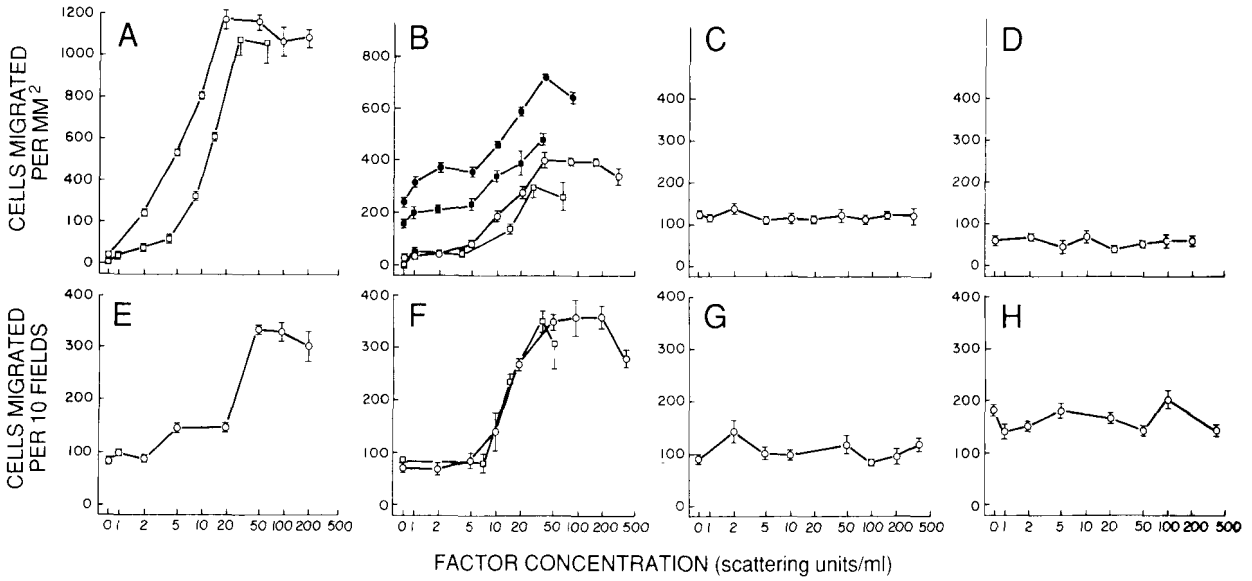

Figure 7. Dose-response curves for purified ras-3T3 and partially purified human smooth muscle scatter factor in quantitative cell migration assays. (A–D) Boyden chamber assays. Assays were performed as described in Materials and Methods. Data are expressed as cells per mm² ± 1 SEM (n = 3). (E–H) microcarrier bead assays. Assays were performed as described in Methods and Materials. Migration is expressed as cells per 10 fields (approximately 0.2 cm²) ± 1 SEM (n = 3). See below for information concerning target cell type, factor source, and incubation time for A–H.

	Target cell type	Factor source	Incubation time (hr)
A.	MDCK epithelium	ras-3T3 (○)	18
	MDCK epithelium	Smooth muscle (□)	18
B.	Calf pulmonary artery endothelium	ras-3T3 (○)	4
	Calf pulmonary artery endothelium	Smooth muscle (□)	4
	Human umbilical vein endothelium	ras-3T3 (●)	4
	Human umbilical vein endothelium	Smooth muscle (■)	4
C.	ras-transformed 3T3	ras-3T3	4
D.	BALB/c 3T3	ras-3T3	4
E.	MDCK epithelium	ras-3T3	18
F.	Calf pulmonary artery endothelium	ras-3T3 (○)	6
	Calf pulmonary artery endothelium	Smooth muscle (□)	6
G.	ras-transformed 3T3	ras-3T3	6
H.	BALB/c 3T3	ras-3T3	18

purification. Smooth muscle-derived SF binds to concanavalin A-Sepharose and can be eluted using α-methyl mannose (9). ras-3T3 SF behaves similarly on concanavalin-Sepharose chromatography, suggesting it might be a glycoprotein.

Similar to the findings of Gherardi, reduction of ras-3T3 SF resulted in appearance of two bands (58 and 31 kDa), with a minor 90-kDa band in some purifications. Thus, SF may be an 85–90-kDa protein consisting of 58- and 31-kDa disulfide-bonded subunits. Alternatively, factor may be cleaved proteolytically and the cleavage fragments held together by disulfide bonds. Both explanations are consistent with the variable 90-kDa band, which could represent a partially reduced or noncleaved factor. These two possibilities (heterodimer vs proteolytic cleavage) have different implications for gene cloning: two structural genes encoding for 58- and 31-kDa peptides vs a single gene encoding for a 90-kDa protein.

Stoker (24) recently reported that partially purified SF stimulates migration of MDCK epithelial cells in Boyden chamber assays. Our findings indicate that the same purified protein which scatters epithelial cells (ras-3T3 SF) also stimulates migration of epithelial and vascular endothelial cells in two quantitative assays (Fig. 7). Partial sequence data suggest this is a new protein not similar to known proteins in the Protein Database. We could not obtain sufficient scatter activity to purify human smooth muscle SF to homogeneity, because smooth muscle strains have limited *in vitro* growth potential. Partially purified smooth muscle SF and purified ras-3T3 SF showed similar dose-response curves in endothelial and MDCK epithelial cell migration assays (Fig. 7). However, the relationship of the

Table III. Basic FGF Stimulates DNA Synthesis in Human Melanocyte Cultures but Scatter Factor Does Not

Addition to culture[a]	[^{3}H]Thymidine uptake[b] (cpm)
None	148 ± 23
dbcAMP (1 m*M*)	190 ± 10
Basic FGF (2 ng/ml)	1,350 ± 86
dbcAMP + basic FGF	19,000 ± 1,000
ras-3T3 SF + dbcAMP (units/ml)	
10	221 ± 21
100	300 ± 49

[a] dbcAMP, dibutyryl cyclic adenosine monophosphate; FGF, fibroblast growth factor (human); ras-3T3 SF, ras-3T3 scatter factor partially purified 1000-fold by cation exchange chromatography. Assays were performed as previously described (23). Cultures were incubated for 3 hr in MEM supplemented with 5 μCi/ml [^{3}H]thymidine and with the indicated additions; radioactivity incorporated into cell DNA was measured.

[b] Values represent mean ± range for $n = 2$ replicate determinations.

two factors cannot be ascertained until each is sequenced completely.

SF, AMF, and MSF are soluble and heat-labile proteins whose primary activity appears to be regulation of cell locomotion. These proteins exhibit higher molecular mass (77, 54, 70 kDa) than well known growth and differentiation factors such as epidermal growth factor, FGF, platelet-derived growth factor, and TGF A and B (6–30 kDa). The three proteins may be cell-specific regulators of mobility, distinct from growth factors, spreading and attachment factors (e.g., fibronectin and vitronectin (25)), intercellular adhesion molecules (26), and metalloproteinases. Their *in vivo* functions are not known, but their activities suggest that they may be involved in developmental processes and/or tissue repair. A role for SF in embryogenesis was postulated (8, 27). Stimulation of epithelial migration by fibroblast-derived SF suggests that SF may be involved in epithelial wound healing. Stimulation of vascular endothelial migration by smooth muscle-derived scatter activity suggests a role in repair of vascular injury. Small gaps in the endothelium are repaired by cell spreading and migration, whereas large gaps are partially filled by migration before the onset of replication (28). Thus, SF(s) may act in conjunction with growth factors to promote vascular repair.

Note added in proof. Subsequent search of amino acid sequences of the four tryptic peptides against the Swiss Prot Database revealed significant homology (86%) with human hepatocyte growth factor. (As of August 8, 1990, HGF has not yet been entered into the NBRF Database.) Determination of the exact relationship between ras-3T3 scatter factor and HGF will require the complete amino acid sequence of scatter factor.

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