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Purification and characterization of biologically active scatter factor from *ras*-transformed NIH 3T3 conditioned medium

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Scatter factor (SF), a glycoprotein produced by cultured fibroblasts, acts *in vitro* on epithelial cells causing separation and increased local motility. In this study, the polypeptide was purified to apparent homogeneity in high yields with conserved biological activity from medium conditioned by *ras*-transformed NIH 3T3 cells, by a three-step procedure involving ammonium sulphate fractionation, cation-exchange and hydroxyapatite chromatography. After purification, SF specific activity increased from ~ 0.3 units/μg in unprocessed conditioned medium to ~ 5 units/ng, and cumulative recovery of biological activity was ~ 38%. Treatment of pure SF with *N*-glycanase resulted in a decreased M_r, but no concomitant effect was observed on biological activity. Proteolytic activity was absent from samples of both partially purified and pure SF. Our biochemical studies showed that SF, which is highly aggregated in low-ionic-strength media, is not aggregated in 0.4 M-salt. Under non-reducing conditions, pure SF migrated as a single stained band at M_r 67000 on SDS/PAGE, and biological activity was eluted from unstained gels with an identical M_r. SF was electrofocused sharply at pI 8.5 with no degradation of activity. From ultracentrifugation studies (under non-aggregating conditions), the sedimentation coefficient of active SF was 3.7 S and f.p.l.c. molecular sieve chromatography indicated a Stokes' radius of 2.95 nm. The calculated M_r from these data was 61400. The appearance of three stained polypeptides of M_r 82000, 57000 and 32000 derived from the M_r-67000 constituent after reduction with mercaptoethanol suggests that SF may be a heterodimer of M_r-57000 and -32000 subunits. Data from protein sequence analysis of the hydroxyapatite-purified protein confirms that SF has sequence identity with both rat hepatocyte growth factor and human fibroblast tumour cytotoxic factor.

INTRODUCTION

Much current interest is centred on the role of autocrine and paracrine cell motility factors in the regulation of cell movement [1–4]. In particular, two groups have recently described experiments with scatter factor (SF), a protein with paracrine action that is secreted by various fibroblast types [5,6] and is also found in human placental tissue [7]. While SF acts *in vitro* on epithelial cells from kidney, bladder, ectocervix and breast by stimulating cell movement, which results in dissociation and cell scattering, no response was reported with fibroblasts [5]. It has been suggested that SF might be involved in embryogenesis or wound healing [5].

SF isolated from mouse NIH 3T3 fibroblasts has been reported to have biologically active M_r values of 62000 [8] and 75000–80000 [7] on non-reducing SDS/PAGE, while under reducing conditions up to three biologically inactive components (M_r 30000, 57000 and 90000) are observed [7,8]. Recently Gherardi & Stoker [9] deduced an *N*-terminal sequence for the reduced M_r-30000 subunit recovered after reverse-phase chromatography and SDS/PAGE. Their data indicated significant sequence identity with a subunit of hepatocyte growth factor (HGF) [10–12], a potent mitogen for mature parenchymal hepatocytes which is implicated in liver regeneration. Interestingly, both SF and HGF have very similar but not identical molecular and biological properties. Furthermore, HGF has 38% amino acid sequence identity with human plasminogen, although neither plasminogen nor plasmin exhibits HGF [10] or SF activity. To determine more detailed molecular and biological properties of SF and its relationship to HGF and other proteins requires purification of sufficient quantities of biologically active protein. Additionally, active labelled purified SF would present a useful probe for elucidating whether the biological mechanism of SF action on epithelial cells is receptor-mediated.

In this report we described a simple and rapid three-step procedure suitable for purification to apparent homogeneity of milligram amounts of biologically active SF from *ras*-transformed NIH 3T3 conditioned medium.

EXPERIMENTAL

Materials

Cation-exchange (Mono S) f.p.l.c. columns, wheat-germ lectin–Sepharose and ampholines were obtained from Pharmacia LKB Biotechnology; hydroxyapatite (HTP; Bio-Gel) and Bio-Rad protein assay reagent were purchased from Bio-Rad Laboratories Ltd.; silver stain kits and *N*-glycanase were from Koch–Light Ltd.; and a BCA protein assay reagent was purchased from Pierce and Warriner (U.K.) Ltd. Pre-stained M_r markers were obtained from Life Technologies Ltd.; SDS/PAGE M_r markers were from Amersham International; and gel-filtration M_r markers and other biochemicals were from Sigma Chemical Co.

Cell culture

MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) foetal calf serum (FCS; Gibco). Cells were subcultured and diluted 1:10 twice weekly. For preparation of serum-free conditioned medium, the *ras*-

Abbreviations used: SF, scatter factor; FCS, foetal calf serum; MDCK, Madin–Darby canine kidney; PBS, phosphate-buffered saline (153 mM-NaCl/3 mM-KCl/10 mM-Na_2HPO_4/2 mM-KH_2PO_4, pH 7.4); HGF, hepatocyte growth factor; DMEM, Dulbecco's modified Eagle's medium; HPT, hydroxyapatite; F-CTF, fibroblast cytotoxic factor.

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transformed D4 clone of NIH 3T3 [5] was grown in DMEM containing 10% (v/v) FCS. 10^8–10^{10} cells per litre were incubated at 37 °C for 3–4 days in Techne stirrers containing 10 g of Biospheres (Whatman, 150–210 μm) per litre. The microcarriers were then washed three times with phosphate-buffered saline (PBS) and incubated with an equal volume of serum- and Phenol-Red-free DMEM for 4 days. The supernatant was harvested by centrifugation, adjusted to 1 mM-NaN_3 and stored at 4 °C.

Bioassay for SF activity

MDCK scattering activity was assayed as described by Stoker & Perryman [13] in 96-well plates using doubling dilutions of test fractions in 0.15 ml of DMEM containing 5% (v/v) FCS. Approx. 3000 MDCK cells were added to each well in 0.15 ml of DMEM/FCS and plates were incubated overnight at 37 °C. Cells were fixed with formol-saline (10% formaldehyde/0.9% NaCl) and stained with Methylene Blue. The titre was defined as the highest dilution at which half-maximal scattering was observed. Units of scatter activity per ml were obtained by dividing the titre by 0.3. On occasions, when there was uncertainty in reading the assay end-point, the root mean square dilution of two adjacent wells was taken as the working titre.

Ammonium sulphate fractionation of conditioned medium

Conditioned medium was precipitated with ammonium sulphate at 60% saturation for 1 h at 4 °C, then centrifuged at 10000 *g* for 30 min. The pellet was resuspended in 50 mM-Mes/0.1 M-NaCl, pH 6.0, and stored at −70 °C until required.

Thawed samples were adjusted to 1 mM-phenylmethanesulphonyl fluoride and dialysed for 18 h at 4 °C against 50 mM-Mes/0.1 M-NaCl, pH 6.0, buffer, followed by centrifugation at 20000 *g* for 30 min at 4 °C. The pellet was discarded and the supernatant passed under a vacuum through a 0.22 μm-pore-size filter previously treated with 2.5% (w/v) BSA in PBS (37 °C) prior to f.p.l.c.

Cation-exchange f.p.l.c.

The dialysed filtered ammonium sulphate fraction was loaded on to a 1 ml Mono S column equilibrated with 50 mM-Mes/0.1 M-NaCl, pH 6.0, at room temperature (23 °C), at a flow rate of 1 ml/min. Bound proteins were eluted from the washed column with a linear gradient of 0.1–1.0 M-NaCl buffer, and 2 ml fractions were collected in tubes containing 110 μl of 0.5 M-Tris base, which adjusted the pH to 7.4.

HPT chromatography

A 10 ml HPT bed volume was packed under gravity and washed with a minimum of 10 column volumes of 3 mM-sodium phosphate buffer, pH 7.4, at 4 °C. SF activity from the cation-exchange column was applied at 1 ml/min (4 °C). After washing the HPT column with 3 mM-sodium phosphate buffer until no absorbance reading was obtained at 280 nm, a linear 0.1–0.6 M-sodium phosphate gradient was applied at 1 ml/min (room temperature) and 1 ml fractions were collected.

Wheat-germ lectin f.p.l.c.

The active HPT-purified samples were dialysed for 18 h (4 °C) against 100 vol. of 30 mM-Hepes/5 mM-$MgCl_2$, pH 7.4, prior to loading at 0.4 ml/min on a 2.5 ml bed of wheat-germ lectin–Sepharose 6MB equilibrated in the same buffer at room temperature. After washing the column (10 column vol.) with equilibration buffer, bound SF activity was eluted in buffer containing 0.5 M-*N*-acetyl-D-glucosamine.

Sucrose density gradient ultracentrifugation

Samples containing SF activity were adjusted to 100 μl with appropriate buffers and applied to linear 10–30% (w/v) sucrose gradient tubes (4.6 ml) containing either a low salt (10 mM-KCl) or a high salt (400 mM-KCl) concentration in 10 mM-Tris/1 mM-EDTA buffer, pH 7.4 (4 °C). After ultracentrifugation at 150000 *g* for 16 h (4 °C), 2-drop fractions were collected from the bottom of sample tubes as previously described [14]. The sedimentation markers ovalbumin (3.7 S) and γ-globulin (7.0 S) were applied to separate gradient tubes, and calculation of sedimentation coefficients was made by reference to these standards.

Gel-filtration f.p.l.c. and estimation of M_r

Chromatography of SF activity was performed on a Superose 12 HR 10/30 f.p.l.c. column (Pharmacia–LKB) equilibrated at room temperature with PBS adjusted to contain 0.4 M-NaCl, pH 7.4, at a flow rate of 0.2 ml/min. Samples (200 μl) were applied and the column was eluted at the same flow rate (fraction volume 0.4 ml). The column was calibrated both before and after chromatography of SF activity using the following marker proteins: cytochrome *c* (M_r 12400), carbonic anhydrase (M_r 29000), BSA (M_r 66000) and yeast alcohol dehydrogenase (M_r 150000). The column void volume (V_0), determined with Blue Dextran 2000, was 7.54 ml. Estimates of approximate M_r for biological activity were made from a plot of V_e/V_0 versus $\log M_r$ [15], where V_e is the elution volume.

From the column elution data, a linear calibration for the Stokes' radius (*a*) was derived, according to the method of Siegel & Monty [16]. The M_r (*M*) of the active SF component was deduced from the combined Stokes'–Einstein–Svedberg equations [15]: $M = 6\pi\eta aNs/(1-\bar{v}\rho)$, where η is viscosity, ρ is the density of the medium, $\bar{v}$ is partial specific volume (assumed to be 0.715 cm^3/g), *s* is the sedimentation coefficient and *N* is Avogadro number.

Electrophoresis

SDS/PAGE of various preparations was performed in 1.5 mm-thick gel slabs containing 8% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel, in 0.1% (w/v) SDS. Reduced samples were boiled for 2 min with 2% (w/v) SDS/5% (v/v) 2-mercaptoethanol [17]. Non-reduced samples were mixed with sample buffer without 2-mercaptoethanol and were not boiled. After electrophoresis, gels were stained with silver stain reagents. Marker proteins used were: myosin (M_r 200000), phosphorylase *b* (M_r 97400), BSA (M_r 69000), ovalbumin (M_r 46000), carbonic anhydrase (M_r 30000) and lysozyme (M_r 14300). In experiments where SF activity was eluted from gels, samples were loaded in 30 mm-wide wells and pre-stained marker proteins were applied to wells at the centre and edges of the gel slab. After electrophoresis, 20 mm-wide sample lanes were cut into 2 mm-deep sections in the M_r region ~ 28000–100000. All slices were incubated at 37 °C for 16 h in 0.5 ml of 50 mM-ammonium carbonate/1 mM-SDS, pH 7.8 [8]. Gel eluates were examined for biological activity in the MDCK cell scatter assay after an initial dilution of 1:16 with PBS.

Electrofocusing

Cation-exchange-isolated SF activity was dialysed against 100 vol. of 30 mM-Hepes/5 mM-$MgCl_2$ buffer at pH 7.4 (4 °C), and adjusted to 5% (w/v) glycerol and 1% (w/v) ampholines (pH 7–9 and pH 9–11, mixed 1:1). The sample was applied to a 50 ml jacketed column containing 2% (w/v) ampholine mixture in a pre-formed linear 0–55% (w/v) sucrose gradient, cooled to 4 °C. Isoelectrofocusing was performed at 300 V (4 °C) for 16 h

as previously described [18]. At the completion of focusing, 30-drop fractions collected from the bottom of the column were assayed for SF activity. The pH of each fraction was measured at 4 °C.

N-Glycanase treatment

Portions of HPT-purified SF (~ 1 μg) were mixed with 2 units of *N*-glycanase (activity 250 units/ml) diluted in 100 mM-ammonium bicarbonate, pH 8.6, in a total volume of 30 μl and incubated at 25 °C for periods of 6 h and 18 h. Control incubations were also performed in the absence of enzyme. At the specified times, the reaction was terminated by freezing at −70 °C. Treated, control and untreated preparations were assayed for SF activity. All samples were analysed by SDS/PAGE.

Assays for proteolytic activity in SF preparations

Samples containing non-denatured SF activity were electrophoresed in SDS/8% polyacrylamide gels containing gelatin previously dissolved at a 1 mg/ml gel mix; trypsin dilutions were applied as positive controls to separate sample wells. At run completion, gels were twice soaked in 2.5% (w/v) Triton X-100 for 15 min at room temperature, before incubation in substrate buffer [50 mM-Tris/5 mM-$CaCl_2$/0.02% (w/v) NaN_3, pH 8.0] for 18 h at 37 °C with gentle agitation [19]. Gels were stained with 0.5% (w/v) Coomassie Blue R in 40% (v/v) methanol/7% (v/v) acetic acid and destained in 40% (v/v) methanol/7% (v/v) acetic acid. Assessment of proteolytic activity in gel-separated components was detected by inspection of the gel for clear unstained bands in the gelatin substrate.

Protein assays

The BCA protein assay and Micro BCA protein assay were used routinely for all preparations, with the exception of samples from wheat-germ lectin f.p.l.c., where *N*-acetyl-D-glucosamine caused excessive interference with the assay. The Bio-Rad (Micro) protein assay, which gives minimal assay interference with *N*-acetyl-D-glucosamine, was used with these samples.

Protein electroblotting and sequencing

Purified non-reduced M_r-67000 SF was electroblotted on to an Immobilon P membrane (Millipore) and stained with sulphorhodamine B [0.005% (w/v) in 30% (v/v) aqueous methanol/0.02% (v/v) acetic acid] for 1 min. The stained band was excised from the washed Immobilon P sheet and covalently immobilized by treatment with 1,4-phenylene di-isothiocyanate and poly(allylamine) [20], before sequencing for 24 cycles using a Milligen 6600 solid-phase sequencer.

RESULTS AND DISCUSSION

Purification

We have devised a simple three-step process which provides substantial quantities of pure SF from *ras*-transformed NIH 3T3 conditioned medium, with retained biological activity and in a form suitable for direct investigation of the biological, molecular and biochemical properties of the motility factor. A previous study described the purification of SF by reversed-phase chromatography [8], but this methodology yielded small quantities of unstable SF. Throughout our work, the biological activity of SF in conditioned medium and other preparations was assessed using the MDCK cell dispersal assay described by Stoker & Perryman [13]. In this laboratory, the specific activity of SF in unprocessed conditioned medium was usually ~ 0.3 unit/μg of protein, although occasionally higher or lower activities were found.

Studies directed towards initial concentration and enrichment of SF indicated that high yields of activity were recovered from conditioned medium by precipitation with ammonium sulphate (Tables 1 and 2) before separation by cation-exchange f.p.l.c. Adsorbed fractionated activity was eluted from a Mono S f.p.l.c. column at 0.49 ± 0.03 M-NaCl (25 experiments) and resulted in overall purification of ~ 1600-fold (Table 2). Qualitative examination of non-reduced cation-exchange-purified material by SDS/PAGE and silver staining confirmed the presence of many contaminants (Fig. 1, track a) in this partially purified preparation.

We found that SF activity was adsorbed in the presence of high salt concentrations (0.5 M) and could be eluted from columns of HPT by sodium phosphate buffers at neutral pH (A. Coffer & J. Fellows, unpublished work). Adsorbed pooled Mono S SF activity was eluted from HPT in the region of 0.25 M-phosphate (Fig. 2). Recovery of applied biological activity from HPT was greater than 70% and the specific activity of pooled active fractions increased from 0.31 units/μg in unprocessed conditioned medium to 5 units/ng in the HPT eluate (Table 2). Data which have shown that only native proteins effectively bind HPT [21] suggest that this step may also confer an additional level of selectivity on our methodology. The use of HPT chromatography resulted in an overall 16000-fold purification of SF (Table 2). Analysis of this product by SDS/PAGE confirmed the presence of a single major silver-stained band (Fig. 1, lane b).

A portion of HPT-purified SF was further isolated by wheat-germ lectin f.p.l.c. (Fig. 3), with recovered specific activity in the pooled 0.5 M-*N*-acetyl-D-glucosamine eluate (Table 3) of ~ 5500 units/μg of protein, this being little different to the activity of the applied material. Although the specific activity appeared to be significantly increased in one fraction from the wheat-germ lectin column (Table 3), no qualitative differences were detected between material in this single fraction and HPT-purified SF by SDS/PAGE and silver staining (Fig. 1, lane d). Similar results were obtained using concanavalin A–Sepharose f.p.l.c., but this procedure resulted in higher losses of non-adsorbed SF activity to the column effluent (A. Coffer & J. Fellows, unpublished work). Our data confirmed that, following cation-exchange f.p.l.c., use of HPT alone provided optimal recoveries of active purified SF.

Characterization of SF

It was important to establish the stability of the biological activity in SF preparations at different stages of purification.

Table 1. Ammonium sulphate precipitation of SF from conditioned medium

Each of five 10 ml portions of conditioned medium from *ras*-transformed NIH 3T3 cells, containing 8.6 mg of protein and 2500 units of SF activity, was brought to the indicated ammonium sulphate saturation as described in the Experimental section. Protein pellets were reconstituted in 1 ml of 50 mM-Mes/0.1 M-NaCl buffer (pH 6.0) before assay.

Ammonium sulphate (% saturation)	Recovery			
	Protein		Biological activity	
	(μg)	(%)	(units of SF)	(%)
25	72	0.83	0	0
30	94	1.09	125	5
40	96	1.12	125	5
50	134	1.56	500	20
60	168	1.95	1750	70

Table 2. Purification of SF from *ras*-transformed NIH 3T3 conditioned medium

			SF				
Step	Volume (ml)	Total protein (mg)	$10^{-3}\times$ Activity (units)	$10^{-3}\times$ Total activity (units)	Specific activity (units/μg)	Recovery (%)	Purification (fold)
Conditioned medium	74000	51200	0.21	15762	0.31	100	1
Ammonium sulphate	1480	1297	6.84	10123	7.80	64	25
Mono S	74	16.2	109	8066	498	51	1603
HPT eluate	10	1.2	593	5930	4945	38	15942
HPT effluent	78	5.2	0	–	–	–	–

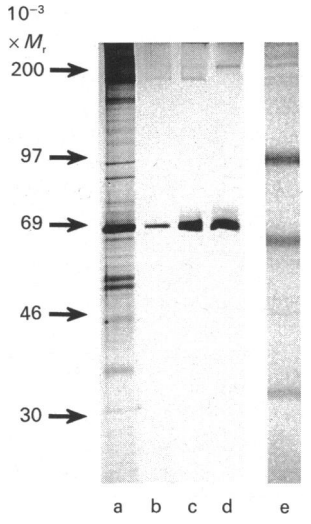

Fig. 1. SDS/PAGE analysis of SF purification

Samples were loaded on a 8% (w/v) polyacrylamide gel containing SDS and electrophoresed (see the Experimental section). At completion, the gel was silver-stained for protein. Tracks: a, Mono S pool, 5 μg of protein; b, HPT pool, ~0.2 μg of protein; c, HPT peak fraction, ~0.3 μg; d, wheat-germ lectin–Sepharose peak fraction 19, ~0.3 μg; e, mercaptoethanol-reduced SF (~2 μg of purified protein) applied to a separate gel.

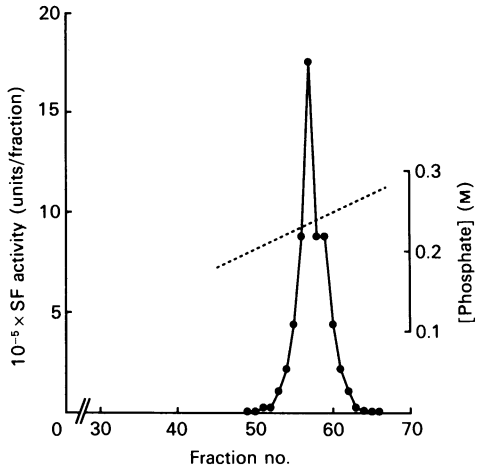

Fig. 2. Purification of SF on HPT

A Mono S f.p.l.c. pool of SF activity (~8×10^6 units) containing 16 mg of protein was applied at a flow rate of 1 ml/min to a 10 ml bed volume of HPT at 4 °C. After extensive washing, the column was eluted at room temperature with a linear gradient (3–600 mM) of sodium phosphate (see the Experimental section). Fractions were assayed for SF activity (●), and the gradient sodium phosphate concentration (---) was determined directly in column fractions by conductivity measurements.

Activity monitored in Mono S preparations showed no change over 12 days at 4 °C or after 60 days at −20 °C. When subjected to heating at 60 °C, a significant fraction (50%) of Mono S f.p.l.c. biological activity in 0.5 M-NaCl/Mes buffer, pH 7.4, was lost within 5 min; this increased to >80% at 30 min and resulted in total inactivation at 60 min. HPT-purified SF activity was stable for 14 days at 4 °C, after which degradation slowly occurred to the extent of loss of 80% of activity at 60 days. Loss of activity was prevented by the inclusion of 0.2% (w/v) BSA, which stabilized SF activity in preparations for 60 days at 4 °C. When HPT-purified SF was stored at −70 °C in the absence of BSA, activity was preserved for 30 days. An inactivation of 50%, which occurred by 60 days at −70 °C, was prevented by inclusion of BSA as described.

Neither partially purified nor HPT-purified preparations exhibited metalloproteinase or serine protease activities when analysed by the SDS/PAGE–gelatin substrate assay of Herron *et al.* [19]. Treatment of non-reduced HPT-purified SF with *N*-glycanase had no effect on biological activity, although the enzyme treatment did result in a decrease in M_r from 67000 to 61000 as judged by SDS/PAGE, the M_r of each component after mercaptoethanol reduction decreasing by 2000–3000-M_r (results not shown). That no effect of partial deglycosylation was observed on biological activity is intriguing and contrasts with other data reported for granulocyte-colony-stimulating factor, one of a family of glycoprotein haemopoietic growth factors, where even partial deglycosylation produced significant increases in biological activity [22].

Analysis of non-reduced HPT-purified SF by SDS/PAGE and silver staining detected a single band at M_r 67000 (Fig. 1, lanes b and c). After reduction of purified SF with mercaptoethanol, three silver-stained bands were observed on SDS/PAGE at M_r 82000, 57000 and 32000 (Fig. 1, lane e). Observed silver staining of reduced SF bands was weaker than that of the non-reduced M_r-67000 band. Confirmation that reduced SF gives rise to only three components was obtained after elution of the non-reduced M_r-67000 component from a polyacrylamide gel.

Re-analysis of the eluted material by SDS/PAGE after mercaptoethanol reduction resolved components of M_r 82000, 57000 and 32000 (results not shown). The molecular size of the non-reduced component is similar to that previously reported for SF isolated from *ras*-transformed 3T3 conditioned medium by reverse-phase chromatography [8], although a higher M_r (~78000) was reported for non-reduced SF from conditioned medium and human placenta [7]. The size of polypeptide components observed under reducing conditions compares fav-

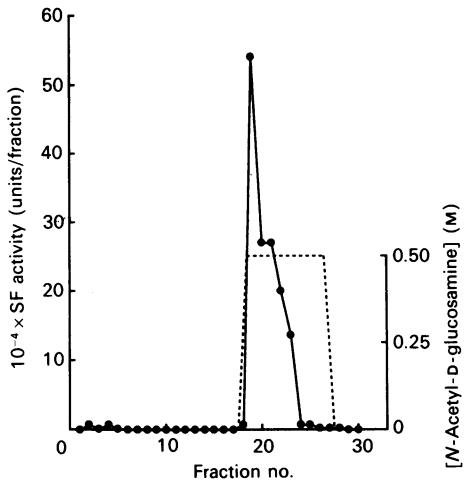

Fig. 3. Isolation of HPT-purified SF by wheat-germ lectin f.p.l.c.

A dialysed sample of pooled HPT-purified SF (~ 700000 units) was applied to a 2.5 ml column bed of wheat-germ lectin–Sepharose as described in the Experimental section. After column washing, SF activity (●) was eluted in buffer containing 0.5 M-*N*-acetyl-D-glucosamine (---). Column recovery data are summarized in Table 4.

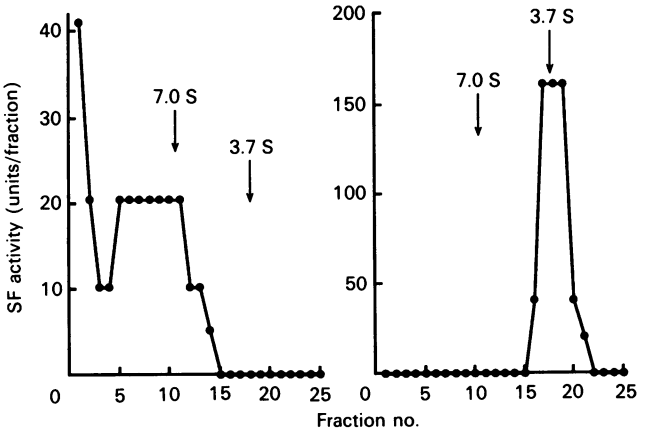

Fig. 4. Sucrose density gradient sedimentation profiles

Portions of Mono S-pooled fractions containing ~ 1400 units of SF in 100 μl were applied to 10–30% (w/v) linear sucrose gradients containing low salt (10 mM-KCl, *a*) or high salt (400 mM-KCl, *b*) concentrations and centrifuged at 150000 *g* for 16 h at 4 °C (see the Experimental section). Fractions collected from the bottom of tubes were assayed for SF activity. Arrows denote the positions of ovalbumin (3.7 S) and γ-globulin (7.0 S) sedimentation markers applied to separate gradients.

Table 3. Further isolation of HPT-purified SF by wheat-germ lectin f.p.l.c.

Approx. 700000 units of SF activity were applied to the column. The purification is relative to the SF specific activity in unprocessed conditioned medium (0.35 unit/mg).

Column fraction no.	Protein (μg)	10^{-3} × Total SF activity (units)	10^{-3} × Specific activity (units)	Purification (fold)
19	6	54.70	9.12	26057
20	5	27.33	5.47	15628
21	5	27.33	5.47	15628
22	4*	20.50	5.13	14000
23	4*	13.65	3.41	10000
Column effluent	16	17.06	1.06	–

* Estimate of ⩾ 4 μg of protein/fraction.

ourably with data from recent reports proposing that SF may be a heterodimer with subunits of M_r 57000 and 32000 [7,8], held together by intrachain disulphide bonds. However, while it has been suggested that a reduced M_r-90000 component, which is seen only occasionally, may arise as a result of incomplete mercaptoethanol reduction of native (M_r 62000) SF [8], a reduced major M_r-82000 component, which is always observed in our preparations, may represent an unprocessed form of SF protein. Reconstitution experiments using inactive subunits prepared from pure reduced SF may resolve those components identified with biological activity. An estimate of molecular size for the biologically active non-reduced SF component was obtained from four experiments in which, following electrophoresis of purified SF preparations, gels were sectioned into 2 mm slices in the M_r region 20000–100000. After buffer elution of gel slices (see the Experimental section), approx. 80% of the total SF activity recovered was located in the eluate from a single gel slice at an M_r of 67000 ± 1500 (results not shown).

Additional physicochemical characteristics of non-reduced SF were obtained from sucrose gradient ultracentrifugation and molecular sieving studies. In low-salt (10 mM-KCl) sucrose gradients, SF activity was highly aggregated; ~ 18% of the applied activity was sedimented as a diffuse shoulder in the region 7–12 S, while > 80% of the SF activity was aggregated and pelleted to the bottom of the tube (Fig. 4*a*). Ultracentrifugation of the same preparation through a high-salt (400 mM-KCl) sucrose gradient produced a more discrete peak of dissociated SF (> 60% activity) sedimenting at 3.7 S (Fig. 4*b*). Non-reduced SF was chromatographed in 400 mM-NaCl on a Superose 12 f.p.l.c. column calibrated for M_r (see the Experimental section). A sharp peak of activity was eluted from the column with an apparent M_r of ~ 50000 (Fig. 5). A calibration for the Stokes' radius was derived from the column elution data for marker proteins used, according to the method of Siegel & Monty [16], and a Stokes' radius of 2.95 nm was extrapolated for SF (Fig. 5, inset). By substituting data for the sedimentation coefficient and Stokes' radius in the combined Stokes'–Einstein–Svedberg equation ($M = 6\pi\eta aNs/(1-\bar{v}\rho)$; see the Experimental section), a calculated M_r value of 61400 was obtained for active non-reduced SF. Molecular parameters transposed in the equation:

$$f/f_o = a\left(\frac{4\pi N}{3\bar{v}M}\right)^{\frac{1}{3}}$$

indicated a frictional coefficient (f/f_o) of 1.14 for non-reduced SF. On the assumption that the degree of hydration for SF is ~ 0.3 g of water/g of protein (a value typical for many proteins), by reference to published tables [23], a frictional coefficient of 1.14 would be consistent with a prolate ellipsoid with an axial ratio of ~ 4:1. As SF is a glycosylated protein ([4]; the present paper), the M_r of the non-reduced M_r-67000 component observed from SDS/PAGE studies may reflect the anomalous behaviour of glycoproteins in SDS/polyacrylamide gels [24].

Under non-denaturing conditions, a major peak of SF activity was electrofocused sharply in a preparative sucrose-stabilized pH gradient at pI 8.5, with a minor component resolved in the region of pI 10.5 (Fig. 6). These data compare favourably with those of

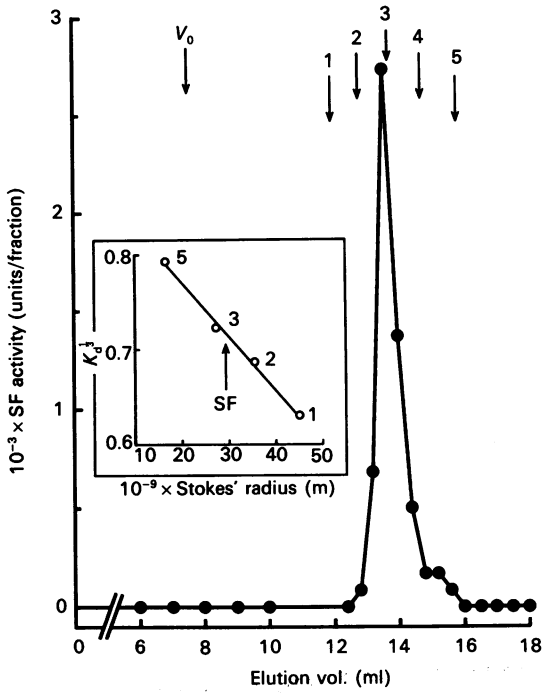

Fig. 5. Analysis of SF activity by Superose 12 f.p.l.c.

After column equilibration with buffer (PBS adjusted to contain 0.4 M-NaCl, pH 7.4), 200 μl of the Mono S SF preparation was applied and the column was eluted (0.2 ml/min) at room temperature. Fractions (0.4 ml) were monitored for biological activity. Arrows indicate elution volumes for M_r markers: 1, alcohol dehydrogenase (M_r 150000); 2, BSA (M_r 66000); 3, ovalbumin (M_r 45000); 4, carbonic anhydrase (M_r 29000); 5, cytochrome *c* (M_r 12400). The column void volume (V_0), determined with Blue Dextran 2000, was 7.5 ml. Inset: correlation of K_d with Stokes' radius of marker proteins. The extrapolated Stokes' radius for SF is indicated at 2.95 nm. Experimental details are described in the text.

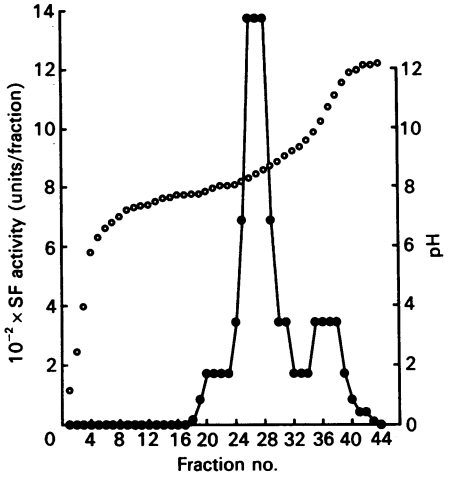

Fig. 6. Isoelectrofocusing of SF biological activity

A portion of dialysed Mono S-partially-purified SF (~ 13000 units) was applied to a sucrose gradient liquid focusing column containing ampholines in the pH range 7–11 and electrofocused (4 °C) for 16 h at 300 V. Column fractions (~ 1 ml) collected at completion were assayed for SF activity (●); pH (○) was measured at 4 °C.

Gherardi *et al.* [8], who eluted a broad zone of SF activity from electrofocused polyacrylamide disc gels in the pI range ~ 8.5–9.5.

Table 4 shows the amino acid sequence obtained from the HPT-purified non-reduced M_r-67000 SF component. SF amino acid residues 1–17 are identical to those of rat HGF, and sequence data also reveal a relationship between SF and human fibroblast cytotoxic factor (F-CTF). Our analysis confirms the identity of residue 13 from mouse SF as tryptophan.

SF activity was previously detected by Stoker & Perryman [13] in conditioned medium from a human embryo lung fibroblast cell line (MRC5), the activity being indistinguishable from that produced by *ras*-transformed NIH 3T3 fibroblasts [8]. A recent report describes the isolation of a tumour cytotoxic factor (F-TCF) from medium conditioned by human embryo lung diploid fibroblasts. F-TCF is cytotoxic for a mouse sarcoma cell line and cytostatic for human epidermoid carcinoma cells [25]. The protein is glycosylated and has almost identical physicochemical properties to SF. Under reducing conditions, F-TCF was separated as three polypeptide chains by SDS/PAGE with M_r ~ 52000, 30000 and 34000, while the native protein separates as two bands (M_r 76000 and 80000). When tested for hepatocyte growth factor activity, F-TCF strongly stimulated DNA synthesis in rat hepatoctytes, confirming the bifunctional activities of this protein. F-TCF was not tested for scatter activity in the MDCK cell assay. However, an examination of *N*-terminal sequence data for the M_r-30000 and -34000 polypeptides [25] indicates that F-TCF has significant sequence identity with SF (Table 4). Also, both internal and *N*-terminal sequence data infer that F-TCF has close identity with SF and HGF (Table 4). cDNA cloning and expression of both SF and F-TCF will resolve the structural and functional relationships between these proteins and HGFs, which together may constitute a group of related cytokines.

In studies defining the biological role of SF, a purified and biologically active protein, isolated as described, would present a potentially useful ligand for establishing the presence, specificity and localization of putative SF receptors on responsive epithelial cells.

Table 4. Amino acid sequence analysis of HPT-purified M_r-67000 SF

Comparison with *N*-terminal sequences from M_r-30000 SF subunit, HGF and F-TCF. For mouse SF, tentative assignments are shown in parentheses. The initial sequencing yield was 8 pmol, with a repetitive sequencing yield of 96% over 24 cycles. X, not identified.

Factor	Amino acid sequence	Reference
Mouse SF (M_r 67000)	VVNGIPTQTTVGWMVSL(K)Y(R)N(K)(T)I	Present study
Mouse SF (*N*-terminal M_r-30000 subunit)	----------X---- - - - - X H -	[9]
Rat HGF (*N*-terminal M_r-34000 subunit)	---------- - - - - K H -	[10]
Human F-TCF (*N*-terminal M_r ~ 30000 subunit)	-------X-NI-X----	[25]

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