ZOOLOGICAL SCIENCE **10**: 925–934 (1993)

A Novel Growth Factor Highly Mitogenic on Immortalized Cells from Rat Serum: Purification and Its Properties

YUMIKO YONEZAWA[1], REIKO HIRAI[2], TETSUO NOUMURA[3] and HIROSHI KONDO[1]

[1]*Department of Experimental Biology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173,* [2]*Section of Tumor Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113, and* [3]*Department of Regulation Biology, Saitama University, 255 Ookubo, Urawa 338, Japan*

ABSTRACT—A growth factor that is highly mitogenic on BALB/c 3T3 cells and exists exclusively in rat and mouse sera was purified from rat serum by heparin affinity chromatography (two times), anion-exchange chromatography and hydrophobic interaction chromatography. The purified factor, rat serum growth factor (R-SGF), was shown as a single band by SDS-gel electrophoresis and its molecular weight was estimated to be 32 kDa. The molecular weight was confirmed by gel filtration chromatography of the purified sample. The DNA synthesis-stimulating activity of R-SGF was lost by treating with protease, mercaptoethanol, acid and heating. R-SGF stimulated DNA synthesis of rat lung fibroblasts only when they were immortalized by going through crisis, whereas it had no effect on normal (mortal) cells tested before the crisis. This biological property of the factor was different from that of well-known heparin-binding growth factors, such as platelet-derived growth factor, acidic fibroblast growth factor and basic fibroblast growth factor, which stimulated DNA synthesis on both the mortal and immortal cells. These results indicate that this factor is a novel growth factor.

INTRODUCTION

Various growth factors in the serum must play important physiological roles throughout mammalian life by regulating cell growth, cell differentiation and cell migration in organs. However, they have not been well studied, except a few major ones such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), because of their existing in small amounts in serum.

In order to analyze serum growth factors, we previously made a comparison study between sera from various mammals, bovines, humans, rats and mice, on their mitogenic activities, and found that rat and mouse sera have greatly high activity as measured by stimulation of DNA synthesis on BALB/c 3T3 cells, whereas fetal bovine serum (FBS) and calf serum (CS) are far less in activity [41]. Bowen-Pope et al. have reported a similar result to ours [8], and other researchers have demonstrated the presence of unknown factors in rat serum, each of which is active to a specific cell type(s) [15, 25]. Through a study on growth factors in rat serum using heparin affinity chromatography, we found the high activity of the serum is caused by a certain heparin-binding growth factor [41]. The factor was found to be present in mouse serum as well, but not in human and bovine sera. We also reported that the mitogenic activity of the factor disappeared when human fetal lung fibroblasts (TIG-1 cells) were used instead of BALB/c 3T3 cells [41]. In the present paper, first we describe the purification of this growth factor, rat serum growth factor (R-SGF), and then study its property of mitogenic activity as well as its

Accepted September 21, 1993
Received August 30, 1993

physicochemical characteristics.

MATERIALS AND METHODS

Animals and preparation of serum

Male Wistar rats of 8–13 months old were obtained from the Supplying Facilities for Aged Animals of the Tokyo Metropolitan Institute of Gerontology. The animals were kept under a SPF condition at 22°C and 55% relative humidity. Serum was collected from those animals as described in our previous paper [21], pooled and stored at −70°C.

Assay of DNA synthesis

DNA synthesis-promoting activity was assayed on BALB/c 3T3 cells by the method described in our previous paper [41]. Briefly, 3T3 cells (2×10^4 cells/ml) were inoculated in Dulbecco's modified Eagle's medium (DME) (pH 7.3) containing 3% CS and kept for 5 h in the medium, which was then replaced by serum-deficient medium (0.2% CS). After 24 h, test samples were added to the cultures, and 16 h later the cultures were pulsed for 3 h with [^{3}H]thymidine, followed by measurement of the incorporation of radioactivity into trichloroacetic acid-precipitable materials.

Purification of R-SGF

Heparin affinity chromatography. Rat serum, after being centrifuged at 12,000 rpm for 20 min, was applied to a heparin-Sepharose CL-6B (Pharmacia) column (22 ml bed volume, 1.5 I.D.×12.5 cm) equilibrated with 10 mM Tris-HCl-0.1% CHAPS-0.7 mM phenylmethylsulfonylfluoride (PMSF) (pH 7.3) (TCPB-1), containing 0.1 M NaCl. Growth factors were eluted at a flow rate of 42 ml/h with the same buffer at first, then with 0.3 M NaCl in TCPB-1, followed by a 0.3–1.5 M NaCl linear gradient in TCPB-1. One ml fractions were collected and the DNA synthesis-stimulating activity of each fraction was determined on BALB/c 3T3 cells. The fractions with the highest activities were pooled, then concentrated and desalted using Centriprep (Amicon Inc., Beverly, MA). Next, the concentrated sample was applied to heparin affinity high performance liquid chromatography on a TSKgel Heparin-5PW column (Tosoh Corp., Tokyo, Japan; 0.75 I.D.×7.5 cm), and eluted at a flow rate of 30 ml/h in the same elution system as that for the preceding heparin column.

Anion-exchange chromatography. A TSKgel DEAE-5PW column (Tosoh Corp.; 0.75 I.D.×7.5 cm) was equilibrated with 10 mM Tris-HCl-0.1% CHAPS-0.5 mM PMSF (pH 6.9) (TCPB-2). The active fractions from heparin HPLC were applied to the column, and material was eluted with the same buffer followed by 0.2 M NaCl in TCPB-2 and then a 0.2–1.2 M NaCl linear gradient in TCPB-2 at a flow rate of 24 ml/h.

Hydrophobic interaction chromatography. Finally, the most active fractions collected from the preceding processes were applied to hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column (Tosoh Corp.; 0.75 I.D.×7.5 cm) having been equilibrated with 0.1 M KH_2PO_4-NaOH-0.05% CHAPS-0.1 mM PMSF (pH 7.0) (KCPB), containing 1.5 M $(NH_4)_2SO_4$. Material was eluted at a flow rate of 18 ml/h by two steps of linear gradient : 1.5 M–0.57 M $(NH_4)_2SO_4$ in KCPB and 0.57 M–0 M $(NH_4)_2SO_4$ in KCPB.

SDS-electrophoresis. The final sample thus purified and the sample partially purified from the second heparin column were applied to polyacrylamide gel electrophoresis according to the method of Laemmli [22]. After electrophoresis, the gel was fixed and stained by a silver staining kit (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Gel filtration chromatography of purified R-SGF

Purified R-SGF (approximately 0.7 ng) was applied to gel filtration chromatography on a TSKgel G2000SW column (Tosoh Corp.; 0.75 I.D.×30 cm) equilibrated with 10 mM Tris-HCl-0.04% CHAPS (pH 7.0), containing 1.0 M NaCl. Material was eluted with the same buffer at a flow rate of 12 ml/h, and 0.2 ml fractions were determined for their activity.

Treatments of purified R-SGF

For the examination of the susceptibility of R-SGF to protease digestion, purified sample (approximately 0.4 ng) was incubated at 25°C for 7 h with 2 μg/ml of non-specific protease (Bacterial,

Type IV, Sigma). The effect of a reducing agent on R-SGF was determined by incubating sample (about 0.5 ng) with 0.1 M 2-mercaptoethanol at 25°C for 3 h. To determine the stability of R-SGF to heat and acid, sample (about 0.5 ng) was heated at 100°C for 5 min, or incubated with 1 M acetic acid for 60 min or 7 h.

Cultures of mortal and immortal cells, and determination of the responses of those cells to R-SGF and other growth factors

Primary cultures of rat fetal lung fibroblasts (RL cells) were prepared from the lung of a Wistar rat fetus (gestational age, 20 days) as described in our previous paper [41], and maintained in DME (pH 7.3) supplemented with 10% FBS and antibiotics. They were subcultured for a long enough period so that the cells went through crisis and became immortalized fully. Cells at 7 population doubling level (PDL) (normal and mortal cells) and cells at 51 PDL (immortalized cells) were used for the determination of DNA synthesis-stimulating activity of purified R-SGF and other well-known growth factors, basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF) and PDGF. With both cells the procedure for DNA synthesis assay was the same as for BALB/c 3T3 cells except that the cell number for both RL cells was 4×10^4/well instead of 2×10^4/well for 3T3 cells. The rat lung fibroblasts as well as BALB/c 3T3 cells were confirmed to be free of mycoplasma contamination [43].

Growth factors

PDGF and bFGF were purchased from Takara Shuzo Co. Ltd., Kyoto, Japan and R & D System, Inc., Minneapolis, MN, respectively. Acidic FGF was kindly donated by Dr. K. Nishikawa of Kanazawa Medical University.

RESULTS

Purification of R-SGF from rat serum

When rat serum was fractioned on a heparin-Sepharose CL-6B column, four peaks which stimu-

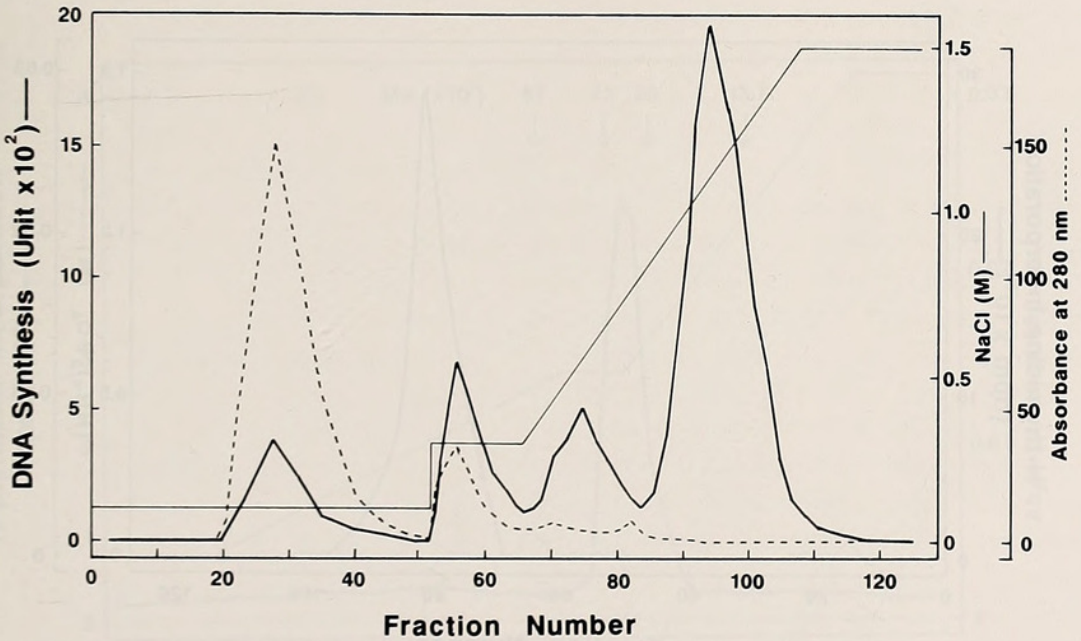

FIG. 1. Heparin-affinity column chromatography of rat serum
Rat serum (about 10 ml) was loaded onto a heparin-Sepharose CL-6B column and eluted at a flow rate of 42 ml/h as described in Materials and Methods. One ml fractions were collected and their absorbance at 280 nm were monitored (dotted line). The mitogenic activity was measured by incorporation of [^{3}H]thymidine into BALB/c 3T3 cells and expressed by units; one unit was defined as the amount of FBS which elicited a half maximal stimulation of DNA synthesis in the same assay procedure (thick solid line). The thin solid line represents the molar concentration of NaCl.

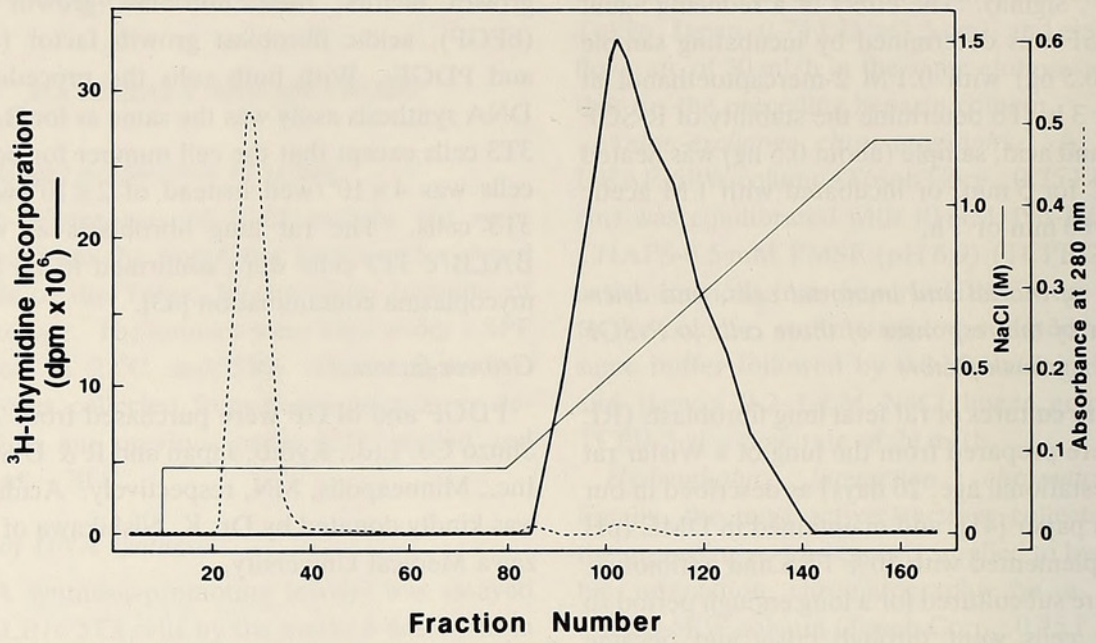

FIG. 2. Ion-exchange chromatography on a DEAE-5PW column
The pooled sample of active fractions from heparin-affinity HPLC was loaded onto a DEAE-5PW column and eluted as described in Materials and Methods. Four hundred microliter fractions were collected and their absorbance at 280 nm was monitored (dotted line). The mitogenic activity of each fraction was measured by incorporation of [^{3}H]thymidine into BALB/c 3T3 cells and expressed as dpm per well (1 ml) in the thick solid line. The NaCl concentration was expressed in the thin solid line.

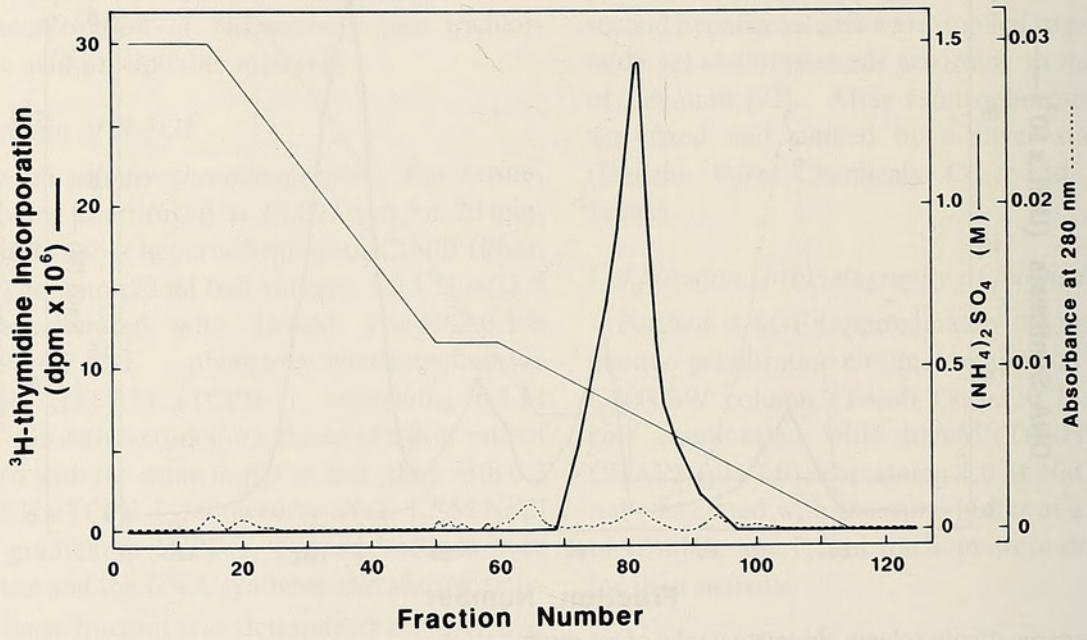

FIG. 3. Hydrophobic interaction chromatography on a phenyl-5PW column.
The pooled sample of active fractions from DEAE column was applied to a phenyl-5PW column and eluted by two steps of $(NH_4)_2SO_4$ linear gradient as described in Materials and Methods. Three hundred microliter fractions were collected and their absorbance at 280 nm and their mitogenic activity on 3T3 cells were determined.

lated the incorporation of [^{3}H]thymidine into BALB/c 3T3 cells were observed (Fig. 1). The fractions of the biggest peak, which eluted at 1.1 M NaCl, were collected and desalted. This step provided more than 3,000-fold increase in the specific activity from the serum level. The sample was then subjected to heparin affinity HPLC on a TSKgel Heparin-5PW column, and only one peak, which eluted at around 1.0 M NaCl, showed mitogenic activity (data not shown). When the fractions of this peak were further fractioned on a TSKgel DEAE-5PW column, mitogenic activity was eluted at around 0.55 M NaCl (Fig. 2). The most active fractions from DEAE column were loaded onto a TSKgel Phenyl-5PW column, and finally purified growth factor was eluted at around 0.35 M $(NH_4)_2SO_4$ (Fig. 3). The material was named rat serum growth factor, R-SGF. About 100 ng of R-SGF was obtained from 100 ml of rat serum by these purification procedures.

Homogeneity of R-SGF

The homogeneity of the final sample (R-SGF) from these purification procedures was analyzed by SDS-PAGE and gel-filtration. On a polyacryla-

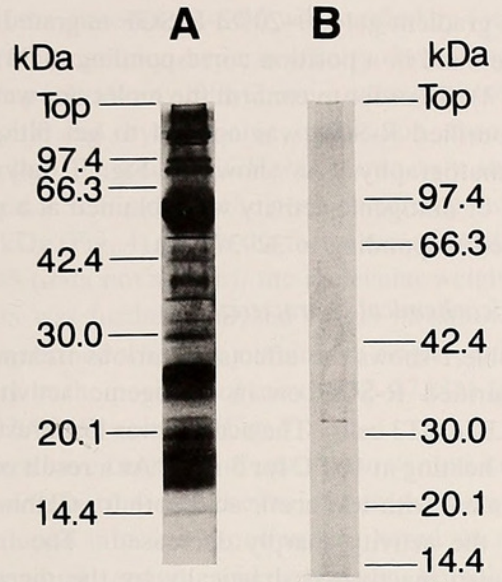

FIG. 4. SDS-PAGE of sample from the second heparin column (A) and purified R-SGF (B)
Samples were dialyzed against 10 mM Tris-HCl (pH 7.2), and the partially purified sample was applied to a 15% polyacrylamide gel (A) and the final preparation (about 10 ng) was to a 10–20% gradient gel (B). The molecular markers were as follows; phosphorylase b (97.4 kDa), bovine plasma albumin (66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa).

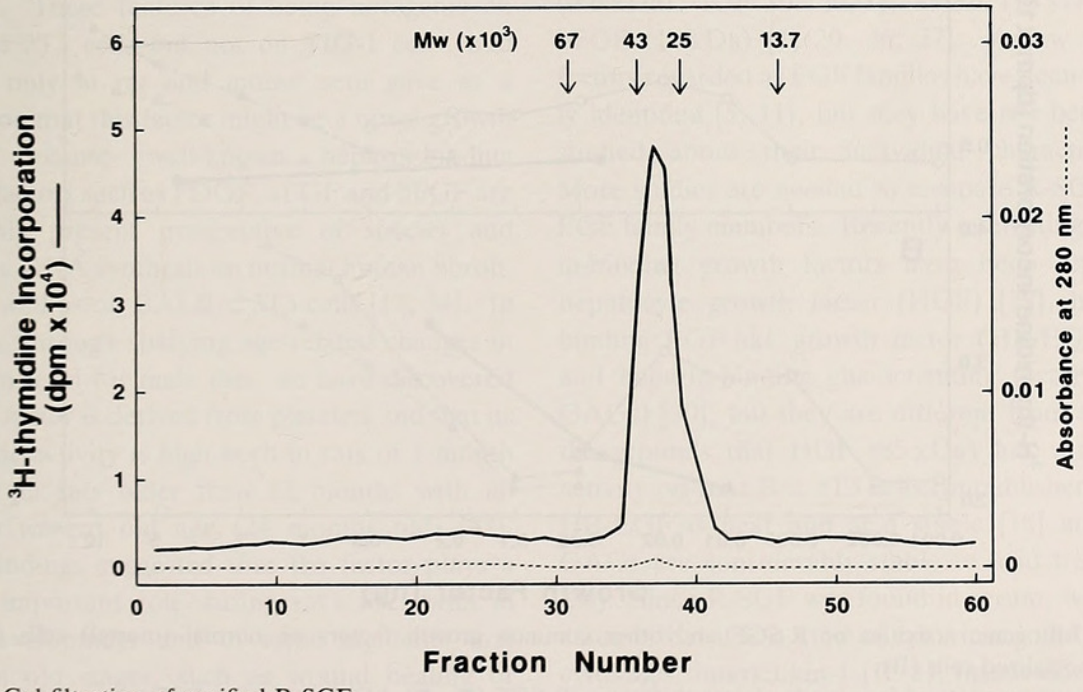

FIG. 5. Gel filtration of purified R-SGF
Purified R-SGF was loaded onto a G2000SW column and eluted as described in Materials and Methods. Two hundred microliter fractions were collected and determined for their mitogenic activity on BALB/c 3T3 cells (thick solid line) and absorbance at 280 nm (dotted line). The molecular weight markers were indicated by arrows: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

mide gradient gel (10–20%) R-SGF migrated as a single band in a position corresponding to 32 kDa (Fig. 4). In order to confirm the molecular weight, the purified R-SGF was applied to gel filtration chromatography. As shown in Fig. 5, only one peak of mitogenic activity was obtained at a position corresponding to 32–37 kDa.

Physicochemical characterization

Table 1 shows the effects of various treatments of purified R-SGF on its mitogenic activity in BALB/c 3T3 cells. The activity was lost drastically by heating at 100°C for 5 min. As a result of the treatment with 1 M acetic acid both for 60 min and 7 hr, the activity sharply decreased. The factor was also inactivated drastically by the digestion with protease, and completely lost its activity by the reduction with 0.1 M 2-mercaptoethanol.

Conditions of cells for responding to purified R-SGF

Rat fetal lung fibroblasts (RL cells) were subcultured to over 60 PDL, and during the course they underwent crisis at 15 PDL (data not shown). As shown in Fig. 6, RL cells which had undergone crises and eventually had become immortalized acquired more intense sensitivity to the well-known growth factors (Fig. 6B) than cells still in mortal state (Fig. 6A), because immortal cells began to elicit DNA synthesis with less amounts of factors and exhibited more amounts of DNA synthesis than mortal cells. Among well-known growth factors tested, bFGF had the highest sensi-

TABLE 1. Effects of various treatments on the mitogenic activity of R-SGF

Treatment	Remaining activity (%)
None	100
100°C, 5 min	4.7
1 M Acetic Acid, 25°C, 60 min	9.9
1 M Acetic Acid, 25°C, 7 hr	6.2
0.1 M 2-Mercaptoethanol, 25°C, 3 hr	0.4
Protease (Bacterial, Type IV), 25°C, 7 hr	5.5

* Values are the means of two test samples.

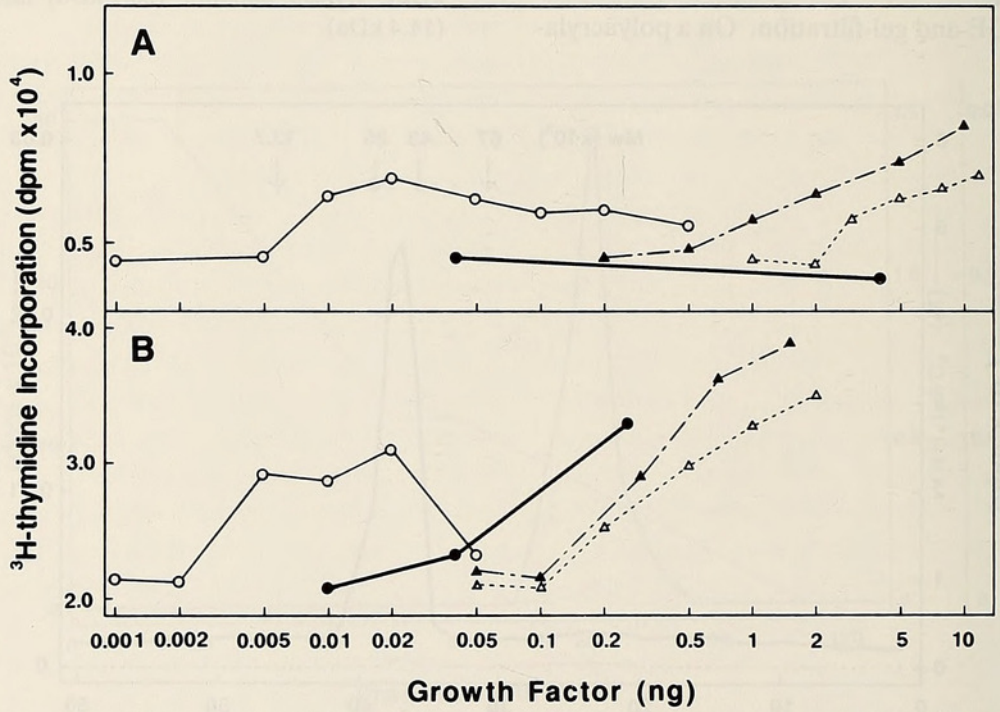

FIG. 6. Mitogenic activities of R-SGF and other common growth factors on normal (mortal) cells (A) and immortalized cells (B)
Various amounts of four kinds of growth factors, R-SGF (●—●), bFGF (○—○), aFGF (▲-·-▲) and PDGF (△···△) were determined for DNA synthesis-stimulating activity on 7PDL (A) or 51 PDL (B) of RL cells (See Materials and Methods). The figures on the horizontal axis indicate the concentration of growth factors (ng/ml well).

tivity to both RL cells, because the amount needed for initiating DNA synthesis stimulation was around 0.01 ng for mortal cells and it shifted to 0.005 ng for immortal cells. The second sensitive was aFGF with the shift from around 0.5 ng for mortal cells to 0.3 ng for immoral cells, followed by PDGF with the shift from around 3 ng to 0.2 ng. To the contrary, R-SGF could not elicit DNA synthesis at all on mortal cells even at the amount of about 4 ng, more than 100 times higher than that needed for immortal cells (around 0.04 ng), although it showed considerably higher mitogenic activity, next to bFGF, on immortal cells. From these results, it is suggested that R-SGF is a novel growth factor exclusively active to immortalized cells.

DISCUSSION

Previously we have reported that rat serum contains a certain heparin-binding growth factor which is highly active on BALB/c 3T3 cells, but not on normal human fetal fibroblasts, TIG-1 cells [41]. We also found that the factor exists in mouse serum besides rat serum, though in less contents, but completely does not in human serum, FBS and CS [41]. These features of being mitogenic on BALB/c 3T3 cells but not on TIG-1 cells, and present only in rat and mouse sera gave us a suggestion that this factor might be a novel growth factor, because well-known heparin-binding growth factors such as PDGF, aFGF and bFGF are commonly present irrespective of species and stimulate DNA synthesis on normal human fibroblasts as well as on BALB/c 3T3 cells [17, 34]. In addition, through studying age-related changes in its serum level for male rats, we have discovered that the factor is derived from platelets and that its mitogenic activity is high both in rats of 1 month old and in rats older than 12 months with an increase toward old age (24 months old) [42]. These findings suggested that the factor plays a certain important role during rat's life: both in early development and in some incidents that occur in old stages, such as wound healing or cancer forming promotion.

In the present paper, we purified the factor, R-SGF, and made a physicochemical characterization of the material. R-SGF was purified by 4 steps of chromatography; heparin-affinity chromatography, heparin affinity HPLC, ion-exchange chromatography and hydrophobic interaction chromatography. Purified R-SGF was shown as a single band on a SDS-plate in the position corresponding to 32 kDa (Fig. 4). Since the factor was susceptible to SDS (data not shown), the molecular weight of 32 kDa was further analyzed by gel filtration. It was confirmed when we obtained a single peak of activity at the position of around 32–37 kDa from the G2000SW column (Fig. 5).

There have been lots of reports on various kinds of growth factors, and the major ones of them can be grouped into five superfamilies based on nucleotide and amino acid sequence homology; PDGF, FGFs, EGF, IGF, transforming growth factor-β (TGF-β) [1, 7, 13, 20, 36, 37]. Among them PDGF and TGF-β are comparable to R-SGF on the grounds that the three are platelet-derived and have similar molecular weight (PDGF, 30 kDa; TGF-β, 25 kDa) [7, 13]. However both PDGF and TGF-β are heat and acid stable [14, 23], whereas R-SGF was highly susceptible to heat and acid treatments as shown in Table 1. Based on molecular weight, R-SGF is not likely to be EGF (0.6 kDa), IGF (0.7 kDa), aFGF (16 kDa) nor bFGF (16 kDa) [1, 20, 36, 37]. A few growth factors regarded as FGF families have been recently identified [5, 11], but they have not been well studied about their individual characteristics. More studies are needed to compare R-SGF with FGF family members. Recently interesting heparin-binding growth factors have been reported, hepatocyte growth factor (HGF) [28], heparin-binding EGF-like growth factor (HB-EGF) [18] and heparin-binding glia-activating factors (HB-GAGs) [30], but they are different from ours on the grounds that HGF (85 kDa) has very little activity on BALB/c 3T3 cells (unpublished data), HB-EGF is heat and acid stable [18] and HB-GAGs are considerably stable to acid treatment [30]. Since R-SGF was found in serum, we could assume that it is a kind of cytokines. Only a few cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) have been reported to be mitogenic on 3T3 cells [19, 39, 40], and the molecular weights of them are all different

from R-SGF's : IL-1 (12–20 kDa), IL-6(21 kDa), TNF (17 kDa) [2, 19, 27]. These findings verified that R-SGF is a novel growth factor different from hitherto structurally investigated growth factors.

In order to clarify the meaning of the previous finding that the factor showed different mitogenic properties between on 3T3 cells and on TIG cells, we used two kinds of cells which were in different stages of cell immortalization: 7 PDL cells (mortal) and 51 PDL cells (immortal). The result shown in Fig. 6 demonstrated that the sensitiveness of cells to the well-known and common growth factors generally increased when cells became immortalized after going through crisis. Although it has been well known that transformed malignant cells have less dependency on serum or growth factors because of their ability to produce autocrine factor(s) [9, 35], there have been almost no reports on a shift in cell sensitivity to growth factors as a result of cell immortalization, except a few reports on the shift in the responses to serum and some hormones [4, 24]. This report is probably the first experimental one exhibiting a change in dose-dependent curves of cell responses to the well-known growth factors caused by cell immortalization. As Denhardt et al. have demonstrated, it might be the case that the establishment of permanent cell lines from primary embryo cells in serum-containing medium reflects the selection of a variant subpopulation of non-preexisting cells [12]. More definite studies are required to clarify the shift in cell responses caused by immortalization.

R-SGF, however, exhibited a completely different feature from that of those factors mentioned above (Fig. 6). The results indicated that R-SGF is a growth factor which stimulates DNA synthesis only when cells get immortalized and can not stimulate that of cells still in mortal state. This unique property of being mitogenic exclusively on immortal cells accounts for the findings in our previous paper [41]. There have been very few reports on growth factors having the similar feature of being exclusively active to immortalized cells. Still Narita has reported that cells spontaneously immortalized from primary liver cells react to a certain autocrine growth factor(s) from the cells, whereas the primary cells do not [29]. The autocrine factor might be a similar kind of one to ours. The presence of these factors could provide a possibility that cells in a specific phase of cellular malignant transformation (normal, immortalized, transformed and metastatic cell phases) require a certain factor(s) specific to the stage. Actually, recent studies seem to support the idea: during malignant transformation, specific growth factors are produced [31, 38], receptors of some growth factors are changed [3, 10, 16] and cell responses to certain factors are changed [6, 26, 32, 33]. R-SGF might be able to offer a great help to studying the cell transformation mechanism.

Since R-SGF exists in sera from normal rats as well as animals with cancer tissues, there is possibility that the factor affects a certain specific type(s) of normal cells *in vivo*. We are now trying to identify the amino acid sequence of R-SGF and planning to clarify its physiological roles *in vitro* and *in vivo*.

ACKNOWLEDGMENT

We wish to thank Dr. M. Takeuchi, Institute for Fermentation, Osaka, for testing cultures for mycoplasma.

REFERENCES

1 Abraham JA, Mergia A, Whang JL, Tumoro A, Friedman J, Hjerrild KA, Gospodarowicz D, Fiddes JC (1986) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233: 545–548

2 Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, Bringman TS, Nedwin GE, Goeddle DV, Harkins RN (1985) Human tumor necrosis factor: production, purification,and characterization. J Biol Chem 260: 2345–2354

3 Allen LE, Maher PA (1993) Expression of basic fibroblast growth factor and its receptor in an invasive bladder carcinoma cell line. J Cell Physiol 155: 368–375

4 Bartek J, Bartkova J, Lalani EN, Brezina V, Taylor-Papadimitriou J (1990) Selective immortalization of a phenotypically distinct epithelial cell type by microinjection of SV40 DNA into cultured human milk cells. Int J Cancer 45: 1105–1112

5 Bates B, Hardin J, Zhan X, Drickamer K, Goldfarb M (1991) Biosynthesis of human fibroblast growth factor-5. Mol Cell Biol 11: 1840–1845

6 Behl C, Bogdahn U, Winkler J, Apfel R, Brysch W, Schlingensiepen KH (1993) Autoinduction of platelet derived growth factor(PDGF) A-chain

mRNA expression in a human malignant melanoma cell line and growth inhibitory effects of PDGF-A-chain mRNA-specific antisense molecules. Biochem Biophys Res Commun 193: 744–751

7 Betsholts C, Johnsson A, Heldin CH, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J (1986) cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. Nature 320: 695–699

8 Bowen-Pope DF, Hart CE, Seifert RA (1989) Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to different classes of PDGF receptor. J Biol Chem 264: 2502–2508

9 Chadwick DE, Lagarde AE (1988) Coincidental acquisition of growth autonomy and metastatic potential during the malignant transformation of factor-dependent CCL39 lung fibroblasts. J Natl Cancer Inst 80: 318–325

10 Cook JR, Wang DY, Chen JK (1993) Nonresponsiveness of an SV40-transformed BALB/c-3T3 cell line to PDGF mitogenicity is correlated with an irreversible reduction in PDGF receptor number. Life Sciences 53: 803–810

11 Delli-Bovi P, Curatola AM, Newman KM, Sato Y, Moscatelli D, Hewick RM, Rifkin DB, Basilico C (1988) Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potential. Mol Cell Biol 8: 2933–2941

12 Denhardt DT, Edwards DR, Mcleod M, Norton G, Parfett CL, Zimmer M (1991) Spontaneous immortalization of mouse embryo cells: strain differences and changes in gene expression with particular reference to retroviral gag-pol genes. Exp Cell Res 192: 128–136

13 Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV (1985) Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature 316: 701–705

14 Deuel TF, Huang JS, Proffitt RT, Baenziger JV, Chang D, Kennedy BB (1981) Human platelet-derived growth factor: purification and resolution into two active protein fractions. J Biol Chem 256: 8896–8899

15 Ferns GAA, Reidy MA, Ross R (1991) Balloon catheter de-endothelialization of the nude rat carotid. Am J Pathol 138: 1045–1057

16 Gitay-Goren H, Halaban R, Neufeld G (1993) Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. Biochem Biophys Res Commun 190: 702–709

17 Gospodarowicz D, Moran JS (1975) Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J cell Biol 66: 451–457

18 Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251: 936–939

19 Hirano T, Taga T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K, Nakajima K, Pymn KH, Kishimoto T (1985) Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFP-2). Proc Natl Acad Sci USA 82: 5490–5494

20 Jaye M, Howk R, Burgess W, Ricca GA, Chiu IM, Ravera MW, O'Brien SJ, Modi WS, Maciag T, Drohan WN (1986) Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. Science 233: 541–545

21 Kondo H, Yonezawa Y, Nomaguchi TA (1988) Effects of serum collected from rats of different ages on in vitro cell proliferation. Mech Ageing Dev 42: 159-172

22 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

23 Lawrence DA, Pircher R, Jullien P (1985) Conversion of a high molecular weight latent TGF-β from chicken embryo fibroblasts into a low molecular weight active TGF-β under acidic conditions. Biochem Biophys Res Commun 133: 1026–1034

24 Lemoine NR, Mayall ES, Jones T, Sheer D, McDermid S, Kendall-Taylor P, Wynford-Thomas D (1989) Characterization of human thyroid epithelial cells immortalized in vitro by simian virus 40 DNA transfection. Br J Cancer 60: 897–903

25 Li ZH, Lu Z, Kirkland JL, Gregerman RI (1989) Preadipocyte stimulating factor in rat serum: evidence for a discrete 63 kDa protein that promotes cell differentiation of rat preadipocytes in primary cultures. J Cell Physiol 141: 543–557

26 Lu C, Kerbel RS (1993) Interleukin-6 undergoes transition from paracrine growth inhibitor to autocrine stimulator during human melanoma progression. J Cell Biol 120: 1281–1288

27 Mizel SB, Mizel D (1981) Purification to apparent homogeneity of murine interleukin I. J Immunol 126: 834–837

28 Nakamura T, Nawa K, Ichihara A, Kaise M, Nishino T (1987) Purification and subunit structure of hepatocyte growth factor from rat platelets. FEBS Let 224: 311–316

29 Narita T (1990) Autocrine growth factor and spontaneous transformation of rat liver epithelial cells. In Vitro Cell Dev Biol 26: 330–334

30 Naruo K, Seko C, Kuroshima K, Matsutani E, Sasada R, Kondo T, Kurokawa T (1993) Novel secretory heparin-binding factors from human

glioma cells (glia-activating factors) involved in glial cell growth: purification and biological properties. J Biol Chem 268: 2857–2864

31 Nemeth J, Goolsby CL (1993) TIMP-2, a growth-stimulatory protein from SV40-transformed human fibroblasts. Exp Cell Res 207: 376–382

32 Nicolson GL, Dulski KM (1986) Organ specificity of metastatic tumor colonization is related to organ-selective growth properties of malignant cells. Int J Cancer 38: 289–294

33 Nicolson GL (1987) Differential growth properties of metastatic large-cell lymphoma cells in target organ-conditioned medium. Exp Cell Res 168: 572–577

34 Phillips PD, Kaji K, Cristofalo VJ (1984) Progressive loss of the proliferative response of senescing WI-38 cells to platelet-derived growth factor, epidermal growth factor, insulin, transferrin, and dexamethasone. J Gerontol 39: 11–17

35 Rodeck U, Herlyn M, Menssen HD, Furlanetto RW, Koprowski H (1987) Metastatic but not primary melanoma cell lines grow in vitro independently of exogenous growth factors. Int J Cancer 40: 687–690

36 Rotwein P, Pollock KM, Didier DK, Krivi GG (1986) Organization and sequence of the human insulin-like growth factor I gene. J Biol Chem 261: 4828–4832

37 Scott J, Urdea M, Quiroga M, Sanchez-Pescador R, Fong N, Seldy M, Rutter WJ, Bell GI (1983) Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. Science 221: 236–240

38 Silletti S, Raz A (1993) Autocrine motility factor is a growth factor. Biochem Biophys Res Commun 194: 446–457

39 Singh JP, Adams LD, Bonin PD (1988) Mode of fibroblast growth enhancement by human interleukin-1. J Cell Biol 106: 813–819

40 Sugarman BJ, Aggarwal BB, Hass PE, Figaro IS, Palladino MA, Shepard HM (1985) Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. Science 230: 943–945

41 Yonezawa Y, Kondo H, Hirai R, Kaji K, Nishikawa K (1992) Species-specific differences in the mitogenic activity of heparin-binding growth factors in the sera of various mammals. Experientia 48: 239–245

42 Yonezawa Y, Kondo H, Noumura T (1993) Age-related changes in the mitogenic activity of heparin-binding growth factors in rat sera. Mech Ageing Dev 69: 179–192

43 Yoshida T, Kawase M, Sasaki K, Mizusawa H, Ishidate M, Takeuchi M (1988) Comparative studies on the sensitivity of three methods for detecting mycoplasmal contamination in cell cultures. Bull Jpn Fed Cul Coll 4: 9–15