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Retina- and Eye-Derived Endothelial Cell Growth Factors: Partial Molecular Characterization and Identity with Acidic and Basic Fibroblast Growth Factors†

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ABSTRACT: Two retina-derived growth factors have been isolated on the basis of their ability to stimulate the proliferation of capillary endothelial cells in vitro. Gas-phase sequence analysis identified the amino-terminal sequence of the major form of the mitogen as being identical with residues 1–35 of bovine basic fibroblast growth factor (FGF). Amino-terminal sequence analysis of the second form identified 28 residues that are indistinguishable from those of brain acidic FGF (residues 1–28). The possibility that these retina-derived endothelial cell growth factors are related to, if not identical with, basic and acidic FGF is supported by observations that they have similar molecular weights (15 000–16 000), similar retention behavior on all steps of chromatography (ion-exchange, heparin–Sepharose), and similar amino acid compositions and that they cross-react with antibodies to basic and acidic FGF. The eye-derived growth factors, like FGF, are potent stimulators of capillary endothelial cell growth in vitro. The results identify the major retina-derived endothelial cell growth factor as indistinguishable from basic FGF and demonstrate the presence of an acidic FGF in the eye. They suggest that at least some of the mitogenic, angiogenic, and neovascularizing activities described as being present in the retina are due to the existence of FGF in this tissue. The implications of this finding on the etiology and pathophysiology of vasoproliferative diseases of the eye are discussed.

Over 30 years ago, Michaelson (1948) proposed the existence of a vasculogenic factor produced by the ischemic retina that could induce neovascularization. Although 20 years were required for direct in vitro and in vivo evidence demonstrating angiogenic and endothelial cell-stimulating activity of the retina (Glaser et al., 1980; D'Amore et al., 1981; Barritault et al., 1981), the original work was pursued by Ashton et al. (1953, 1954) and predicted the involvement of the putative vasculogenic factor in the etiology of diabetic retinopathy, retinal fibroplasia, and retinal vein occlusion.

In recent years several groups have sought to isolate and identify putative angiogenic factors on the basis of various tests for their biological activity. They have been detected and purified to various degrees in extracts of retina (Glaser et al., 1980; Barritault et al., 1981; D'Amore & Klagsbrun, 1984; Courty et al., 1985), cartilage (Folkman et al., 1979; Sullivan & Klagsbrun, 1985), tumors (Shing et al., 1984), platelets (Clemmons et al., 1983), macrophages (Polverini et al., 1977), brain (Maciag et al., 1984; Thomas et al., 1985), and pituitary (Gospodarowciz & Moran, 1976; Esch et al., 1985a). Insufficient purification of these factors, however, has until recently precluded an analysis of their possible homologies to structurally defined growth factors.

Using the knowledge obtained from our recent characterization of basic pituitary fibroblast growth factor (FGF) (Böhlen et al., 1984; Esch et al., 1985a), an angiogenic factor in its own right (Gospodarowicz et al., 1979; Esch et al., 1985a), we have isolated and identified the agents present in the retina that are responsible for the stimulation of endothelial cell growth. In this report, we demonstrate their identity with basic (Böhlen et al., 1984; Esch et al., 1985a) and acidic (Böhlen et al., 1985; Thomas et al., 1984, 1985; Esch et al.,

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Table I: Purification of Acidic and Basic FGF from Bovine Retina[a]

protein purification step	amount recovered (ng/mL)	total ED_{50} (units × 10^6)[b]	recovery of activity (%)[d]	biological factor	x-fold purification
crude extract	7680	1.2×10^3	6.4	100	1
carboxymethyl-Sephadex					
unadsorbed fraction	5265	30×10^3	0.175	2.7	
0.15 M NaCl	705	30×10^3	0.023	0.35	
0.6 M NaCl	320	30	7.62	119	40
heparin–Sepharose					
unadsorbed fraction	312	NA[c]			
1 M NaCl (fractions 38–42)	0.040	4	0.01	0.15	300
2 M NaCl (fractions 52–56)	0.202	0.030	6.73	105	40000

[a] Values are for 1000 retinas (wet weight 610 g). [b] Defined as the concentration of the retina-derived growth factor preparation required to give a 50% maximal response. [c] NA, not active. [d] One unit of activity is defined as the quantity required to give half-maximal stimulation of cell proliferation.

1985b) FGF. The implications of this finding in the understanding and management of vasoproliferative diseases of the eye are discussed.

Experimental Procedures

Extraction and Isolation of Retinal Endothelial Cell Growth Factors. Two batches of frozen bovine retinas (1059 and 1000 retinas) were obtained from J. R. Scientific (Woodland, CA) and processed exactly as described (Gospodarowicz et al., 1984, 1985a,b) except for the omission of the ammonium sulfate precipitation. The crude extract with or without dialysis was applied to a column of carboxymethyl-Sephadex C50 (3 × 20 cm) that had been equilibrated with 0.1 M sodium phosphate, pH 6.0. The column was extensively washed with 0.1 M sodium phosphate (pH 6.0) and the sample eluted by the sequential addition of 0.15 and 0.6 M NaCl in 0.1 M sodium phosphate (pH 6.0) as described earlier (Böhlen et al., 1984).

The majority of the biological activity eluted with 0.6 M NaCl (Table I) and was directly loaded onto a heparin–Sepharose affinity column (1.6 × 5 cm, bed volume 10 mL) that had been equilibrated at room temperature with 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.0) and 0.6 M NaCl. The column was subsequently washed with 10 mM Tris-HCl (pH 7.0) and 0.6 M NaCl, until the absorbency of the eluate at 280 nm became negligible. The adsorbed proteins were then eluted sequentially by the stepwise addition of 0.85, 1, and 2 M NaCl in 10 mM Tris-HCl (pH 7.0).

Final separation of the microheterogeneous forms of the retina-derived growth factors was performed by reverse-phase high-performance liquid chromatography (HPLC) using a semipreparative C_4 column (25 × 0.46 cm, 5-μm particle size, 330-Å pore size) and a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA).

Structural Characterization. Molecular weight determination was performed by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis as described (Gospodarowicz et al., 1984, 1985a,b). Gels were fixed and stained by using the Bio-Rad silver nitrate staining kit, according to the manufacturer's instructions. Amino acid analyses were performed on a Liquimat III analyzer (Kontron, Zurich, Switzerland) equipped with an *o*-phthalaldehyde fluorescence detection system and a proline conversion accessory (Böhlen & Schroeder, 1982). Amino acid sequence analyses of the unmodified, HPLC-purified growth factors were performed with an Applied Biosystems gas-phase microsequenator (Esch, 1984).

Cell Proliferation Assays. The biological activity of the column fractions and purified samples was determined with capillary endothelial cells derived from bovine brain or with vascular endothelial cells derived from bovine aortic arch (Gospodarowicz & Moran, 1976; Gospodarowicz et al., 1985c). Ten-microliter aliquots of the appropriate dilution of each sample were added to the dishes on days 0 and 2. After 5 days in culture, triplicate plates were trypsinized and cell densities determined with a Coulter counter.

Radioimmunoassay. The radioimmunoassay (RIA) for bovine pituitary basic FGF was developed by using antibodies generated in rabbits against synthetic [Tyr^{10}]FGF (1–10) as described (Baird et al., 1985a). The antisera recognize native basic FGF on an equimolar basis and are capable of immunoneutralizing FGF in vitro (Böhlen et al., 1984). They show less than 0.1% cross-reactivity with acidic FGF in the RIA.

Results

Isolation of Retina-Derived FGF. The distribution of bioactive materials between the various purification steps is shown in Table I. When the crude extract of retina was submitted to batch adsorption elution on carboxymethyl-Sephadex C50, only 2.7% of the initial activity was not retained on the column. The major portion of the bioactive material (119%) was recovered in the 0.6 M NaCl eluate. Further purification of the retina-derived growth factor was achieved by heparin–Sepharose affinity chromatography. Most of the protein (>99%) loaded onto the column was unadsorbed and was detected in the unadsorbed fractions (Figure 1A). This unadsorbed material had little of the original biological activity (0.01%). Stepwise elution of the column with 0.85 M NaCl followed by 1 M NaCl revealed a small protein peak with some of the original biological activity (0.15%) that we termed α-retina-derived growth factor (αRDGF). Further elution with 2 M NaCl revealed the presence of a β-retina-derived growth factor (βRDGF) that accounted for 105% of the original activity loaded onto the column and corresponded to a well-defined protein peak.

In Figure 1B, the biological activity of each step of purification including the pooled 1 M NaCl (αRDGF) fractions and 2 M NaCl (βRDGF) fractions is presented. The αRDGF was 8 times more potent than the sample loaded on the column with an ED_{50} at 4 ng/mL. The preparation of βRDGF was 2000 times more potent than the sample loaded on the column, with maximal stimulation of cell proliferation occurring at 200 pg/mL and half-maximal response at 30 pg/mL. The yield of each of the retina-derived growth factors ranged from 66 to 80 μg/kg for αRDGF and from 200 to 331 μg/kg for βRDGF. Molecular weight estimation by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that αRDGF had a molecular weight of ~15 000, while βRDGF had a molecular weight of ~16 500 (not shown). In parallel lanes, their migration was exactly that of brain acidic FGF and brain and pituitary basic FGF, respectively. Similar patterns of migration were observed with or without reduction.

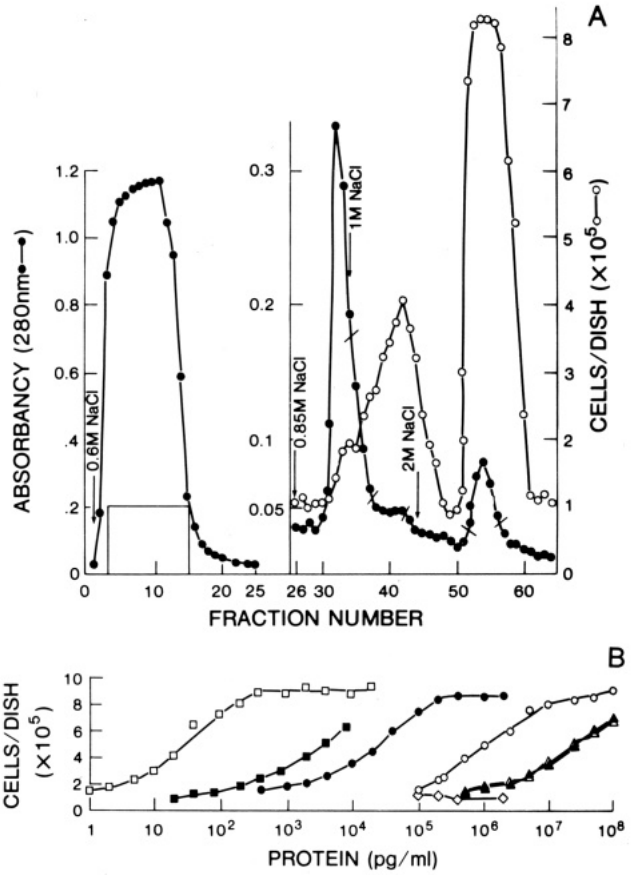

FIGURE 1: Purification of retina-derived growth factors by heparin–Sepharose affinity chromatography. (A) The partially purified preparation (0.6 M NaCl eluate from the carboxymethyl-Sephadex column, 82 mL, 3.9 mg/mL) was chromatographed on a heparin–Sepharose column, as described under Experimental Procedures. Fractions of 9 mL were collected during sample loading and column washing. Aliquots of pooled fractions 3–15 were diluted 5-fold with Dulbecco's modified Eagle's medium (DMEM)/0.5% bovine serum albumin (BSA), and 10-μL aliquots containing 3.9 μg of protein were added to low-density BBC cell cultures. The column was eluted stepwise with 0.85, 1, and 2 M NaCl in 10 mM Tris-HCl, pH 7.0, as described in the text. Fractions (1.4 mL) were collected, and aliquots of the 0.85 and 1 M NaCl eluates were diluted 25-fold in DMEM/0.5% BSA. Fractions collected during the course of the 2 M NaCl elution were diluted 200-fold prior to the bioassay, and 10-μL aliquots were added to the cells. (B) Mitogenic activities of fractions from various purification steps. The crude extract (○) and pools of the carboxymethyl-Sephadex unadsorbed fraction (△), the 0.15 M NaCl eluate (▲), and the 0.6 M NaCl fractions (●) were tested in concentrations ranging from 1 to 100 μg/mL. Pools obtained from the heparin–Sepharose fractions eluting as unadsorbed (◇) or in the 1 M NaCl (αRDGF, ■) or the 2 M NaCl eluate (βRDGF, □) were added to the cells in the concentrations indicated.

Reverse-phase HPLC of αRDGF is shown in Figure 2. Like brain acidic FGF (Böhlen et al., 1985), this retina-derived growth factor requires somewhat higher concentrations of acetonitrile to elute from the column than basic FGF. Final purification of the bioactive peak is presented in panel B of Figure 2. Both peaks of UV absorbance had full biological activity and were used for the partial characterization (see below).

Reverse-phase HPLC of βRDGF revealed that, as in extracts of brain (Gospodarowicz et al., 1984) and kidney (Baird et al., 1985b), there is considerable microheterogeneity of the mitogen (Figure 3). Yet all peaks eluting between 40 and 80 min were biologically active. There were three obvious peaks of activity that corresponded with the three major peaks of basic FGF immunoreactivity as measured by RIA. Final purification of each of the active fractions (Figure 3B–D)

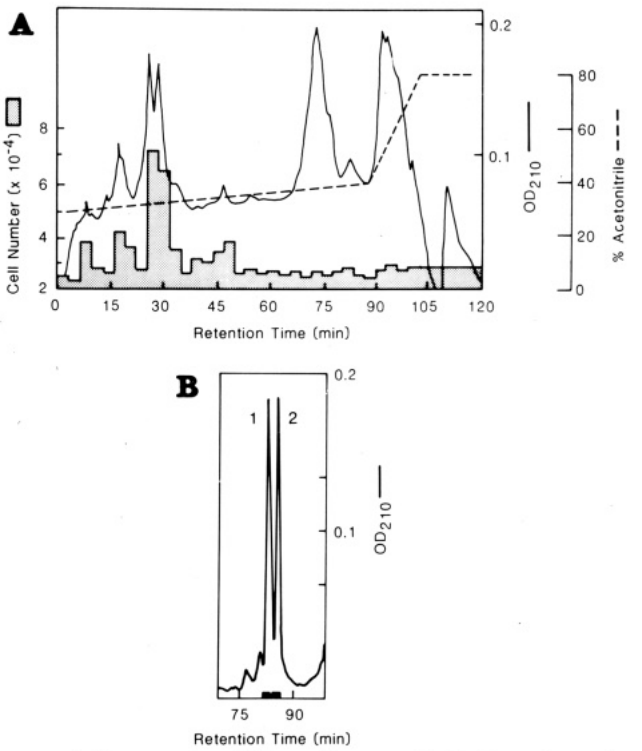

FIGURE 2: Reverse-phase high-performance liquid chromatography of αRDGF. Bioactive fractions eluting with the 1 M wash of the heparin–Sepharose affinity column were pumped directly into a Vydac C_4 semipreparative column. Protein was eluted with a gradient of 30–39% acetonitrile in 0.1% TFA. Aliquots of the column fraction (1 μL) were diluted 10-fold with culture medium and added to low-density aortic arch endothelial cells. The major peak of activity was rerun on the same column as shown in panel B. The peak fractions were taken for amino acid analysis and amino-terminal sequence analysis. The second peak was used for amino-terminal sequence analysis.

provided the necessary resolution to permit a partial chemical analysis of each form (see below).

Characterization of αRDGF and βRDGF. Amino acid analyses of the two microheterogeneous forms of αRDGF isolated from the 1 M NaCl eluate of the heparin–Sepharose affinity column are presented in Table II. These results established the similarities between this factor and acidic FGF characterized form bovine brain. Amino-terminal sequence analyses (Table III) confirmed these results and identified the first 28 residues as being indistinguishable from the sequence of bovine brain acidic FGF (residues 1–28). Further similarities between αRDGF and acidic FGF were established by positive immunoblotting with antibodies raised against a synthetic replicate [Tyr^{69,87}]FGF (69–87) (results not shown). These antibodies cross-react with homologous sequences common to acidic and basic FGF (Esch et al., 1985b).

Amino acid analyses of the three forms of βRDGF established that they were very similar both to each other and to basic pituitary FGF (Table II). Amino-terminal sequence analysis of the first 35 residues of the major form of βRDGF identified 100% homology with basic pituitary FGF (residues 1–35), indicating that the retina-derived endothelial cell growth factor was closely related to, if not identical with, basic FGF (Table III). These results were confirmed by positive immunoblotting with antibodies raised against synthetic replicates of [Tyr^{30}]FGF (30–50) and [Tyr^{69,87}]FGF (69–87) (results not shown).

Discussion

The results presented here identify the endothelial cell growth-promoting activities in the retina as being indistin-

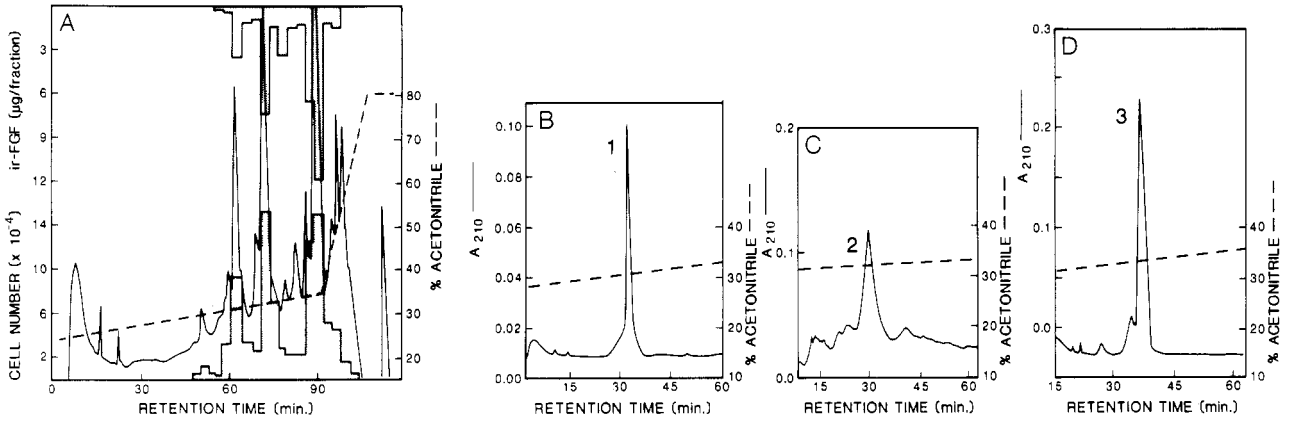

FIGURE 3: Reverse-phase high-performance liquid chromatography of βRDGF. Bioactive fractions eluting after the 2 M wash of the heparin–Sepharose affinity column were pumped directly into a Vydac C_4 semipreparative column. Protein was eluted with a gradient of 24–34% acetonitrile in 0.1% TFA. Aliquots of column fractions (0.1 μL) were added to low-density aortic arch endothelial cells, and each of the three major peaks of activity was rerun on the same column as shown in panels B–D. Peak fractions were taken for amino acid analysis and immunoblotting. Amino-terminal sequence analysis was performed on 500 pmol of the material shown in panel D.

Table II: Amino Acid Composition of Retina-Derived Growth Factors[a]

	αRDGF[d]		brain acidic FGF[e]		βRDGF[b]			
amino acid	peak 1	peak 2	Thomas et al.	Böhlen et al.	peak 1	peak 2	peak 3	pituitary basic FGF[c]
Asx	12.2	12.4	12.9	14.4	11.3	11.5	11.7	12
Thr	6.9	6.8	7.9	7.9	3.7	4.0	3.9	4
Ser	8.3	8.2	9.0	9.1	9.2	9.7	9.2	10
Glx	15.3	14.7	15.1	14.3	12.2	12.0	11.8	12
Pro	6.6	6.7	6.3	5.5	10.6	10.3	10.9	10
Gly	13.6	13.9	13.1	12.2	16.3	16.2	14.6	15
Ala	4.9	4.8	4.2	4.3	9.5	9.2	10.0	9
Cys	3.1	3.1	4.3	3.3	4.6	4.0	4.1	4
Val	4.7	4.5	3.8	4.2	5.8	6.1	6.2	7
Met	1.1	1	0.8	0.9	2.2	2.2	2.3	2
Ile	5.4	5.1	5.2	4.9	2.9	2.9	2.8	4
Leu	18.4	18.9	17.3	18.0	13.0	13.0	13.5	13
Tyr	7.0	6.6	6.8	6.5	6.9	7.1	7.5	7
Phe	6.1	6.7	6.4	7.4	8.2	7.9	8.4	8
His	5.1	5.4	5.0	5.2	2.9	3.0	2.9	3
Trp	0.7	0.6	1.1	1.0	0.8	0.8	1.1	1
Lys	13.0	13.0	12.0	11.8	14.5	14.4	13.9	14
Arg	5.8	5.5	5.3	5.0	11.1	11.2	11.1	11

[a] Values are the mean of duplicate determinations and were obtained from 18-h hydrolysis at 110 °C using 5–10 pmol of protein per analysis. [b] Peptides were purified from the 2 M NaCl eluate of the heparin–Sepharose affinity column as shown in Figure 3B–D, respectively. [c] Values were obtained from the primary sequence of basic pituitary FGF as determined by Esch et al. (1985a). [d] Peptides were purified from the 1 M NaCl eluate of the heparin–Sepharose affinity column as shown in Figure 2B. [e] Values were obtained from Thomas et al. (1984) and from Böhlen et al. (1985) and corrected for a 140 amino acid composition for direct comparison.

guishable from those of acidic and basic FGF. All of the antibodies that have been raised against synthetic fragments of basic FGF cross-react with the major form of the eye-derived mitogen (βRDGF). This growth factor also shares similar molecular weight and similar behavior on all steps of chromatography to those of basic FGF. The amino acid compositions of βRDGF are statistically indistinguishable from those of basic FGF, and its amino-terminal sequence is identical with that of basic FGF.

We have also identified a mitogen that appears indistinguishable from acidic FGF. αRDGF has similar molecular weight and similar behavior on all steps of chromatography to those of acidic FGF, and the amino acid compositions of the two are statistically indistinguishable. Primary sequence analyses of the amino terminus of the mitogen identified the first 28 residues, which are all identical with those of acidic FGF (residues 1–28).

Because both retina-derived growth factors are potent stimulators of endothelial cell growth in vitro, and in view of the capacity of acidic and basic FGF to induce angiogenesis in vivo (Gospodarowicz et al., 1979, 1985a,b; Esch et al., 1985a; Thomas et al., 1985), it seems likely that the molecules identified here are the vasculogenic factors first proposed by Michaelson (1948).

The procedure used to isolate retinal FGF, thé retina-derived angiogenic factor, differed from that already described for other tissues (Esch et al., 1985a; Gospodarowicz et al., 1984, 1985a,b; Baird et al., 1985b) by using a direct chromatography of the crude extract (with or without dialysis) on carboxymethyl-Sephadex without a prior precipitation with $(NH_4)_2SO_4$. The two-step procedure, which consists of batch elution from the ion-exchange column followed by heparin–Sepharose affinity chromatography, led to rapid (less than 24 h) isolation of the growth factors. This last purification step is itself responsible for a 2000-fold purification factor of βRDGF. Although microheterogeneity of the sample is detected on reverse-phase HPLC, the major growth factor purified by heparin–Sepharose affinity chromatography has full intrinsic activity (ED_{50} = 30 pg/mL) without the need for further purification (and the inherent acid-mediated loss of biological activity). The high degree of recovery of the growth factor (105%) indicates that inhibitory substances, which are known to interfere with its bioactivity in crude extracts (Felton et al., 1979; Raymond & Jacobson, 1982), have been eliminated.

Table III: Amino-Terminal Sequence of Retina-Derived Growth Factors

residue no.	βRDGF peak 3[a]	basic FGF[b]	αRDGF peak 2[c]	acidic FGF[d]
1	Pro (139)	Pro	Phe (36)	Phe
2	Ala (112)	Ala	Asn (29)	Asn
3	Leu (167)	Leu	Leu (20)	Leu
4	Pro (88)	Pro	Pro (23)	Pro
5	Glu (50)	Glu	Leu (17)	Leu
6	Asp (46)	Asp	Gly (24)	Gly
7	Gly (79)	Gly	Asn (20)	Asn
8	Gly (81)	Gly	Tyr (16)	Tyr
9	Ser (36)	Ser	Lys (16)	Lys
10	Gly (36)	Gly	Lys (14)	Lys
11	Ala (67)	Ala	Pro (12)	Pro
12	Phe (51)	Phe	Lys (9)	Lys
13	Pro (62)	Pro	Leu (14)	Leu
14	Pro (86)	Pro	Leu (15)	Leu
15	Gly (66)	Gly	Tyr (15)	Tyr
16	His (15)	His	X	Cys
17	Phe (67)	Phe	Ser (7)	Ser
18	Lys (51)	Lys	Asn (10)	Asn
19	Asp (65)	Asp	Gly (11)	Gly
20	Pro (22)	Pro	Gly (16)	Gly
21	Lys (5)	Lys	Tyr (8)	Tyr
22	Arg (18)	Arg	Phe (7)	Phe
23	Leu (34)	Leu	Leu (11)	
24	Tyr (17)	Tyr	Arg (12)	
25	X	Cys	X	
26	Lys (15)	Lys	Leu (6)	
27	Asn (32)	Asn	Pro (6)	
28	Gly (13)	Gly	Asp (2)	
29	Gly (41)	Gly		
30	Phe (8)	Phe		
31	Phe (15)	Phe		
32	Leu (19)	Leu		
33	Arg (15)	Arg		
34	Ile (15)	Ile		
35	His (4)	His		

[a] Sequence data were obtained with 500 pmol of the retina-derived growth factor purified from the 2 M eluate of the heparin–Sepharose column and shown in Figure 3D. Yield (picomoles) is shown in parentheses. The initial yield was 28%, with an average repetitive yield of 92.6%. [b] Primary sequence of basic FGF was obtained from Esch et al. (1985a). [c] Sequence data were obtained with 60 pmol of the retina-derived growth factor purified from the 1 M eluate of the heparin–Sepharose column and shown in Figure 2B (second peak). Yield (picomoles) is shown in parentheses. The initial yield was 41.1%, with an average repetitive yield of 94.8%. [d] Primary sequence of acidic FGF was obtained from Esch et al. (1985a), Böhlen et al. (1985), and Thomas et al. (1985).

In contrast with the results of others (D'Amore & Klagsbrun, 1984), we have been unable to demonstrate larger amounts of the acidic FGF (αRDGF) than of its basic counterpart (βRDGF). With the isolation procedure described here, the amount of acidic FGF is less than 0.15% of the bioactivity detected in the crude extract. While this could be due to the low biological potency of acidic FGF (Esch et al., 1985a; Böhlen et al., 1985), there remains the possibility that different protocols of extraction may account for these apparent discrepancies. The protocol used by others (Glaser et al., 1980; D'Amore et al., 1981; D'Amore & Klagsbrun, 1984) starts with conditioned medium collected from a 2–4-h incubation of dissected retinas in a physiological buffer (pH 7). While in our hands this procedure does generate a basic RDGF (unpublished results), the use of isoelectric focusing (D'Amore & Klagsbrun, 1984) as the first step of fractionation effectively precludes the detection, purification, and isolation of the cationic growth factor.

The results presented here establish the identity of retina-derived growth factors (RDGF) as being indistinguishable from acidic and basic FGF. They also add to the list of tissues such as brain (Gospodarowicz et al., 1984), kidney (Baird et al., 1985b), adrenal (Gospodarowicz et al., 1985b), corpus luteum (Gospodarowicz et al., 1985a), macrophage (Baird et al., 1985d) and tumors (Baird et al., 1985e) that have now been clearly demonstrated as containing FGF. The results also suggest that the presence of "endothelial and fibroblast growth factor activity" in other tissues [reviewed by Baird et al. (1986)] may also be due to the local presence of acidic and/or basic FGF.

With the identification of FGF as the vasculogenic factors postulated by Michaelson (1948), it is interesting to speculate on the physiological and pathophysiological implications of this finding. The effects of FGF on fibroblasts as well as on endothelial cells (Gospodarowicz & Moran, 1976; Gospodarowicz et al., 1985c) may explain the association of fibrosis with the neovascularization that is seen in conditions of retrolental fibroplasia and diabetic retinopathy. The possibility that either or both forms of FGF may be involved in the etiology of vasoproliferative diseases of the eye is an attractive hypothesis that emphasizes the importance in expanding these results. The availability of large amounts of the pure growth factor and a defined chemical structure has already made it possible to apply the sequence-specific RIA for its measurement in ocular fluid (Baird et al., 1985c) and should permit the development of antagonists for use in biological and clinical studies.

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SUPPLEMENTARY MATERIAL AVAILABLE

Copies of the $NaDodSO_4$–polyacrylamide gels and immunoblots described in the text (2 pages). Ordering information is given on any current masthead page.

Registry No. Fibroblast growth factor, 62031-54-3.

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Specific Interaction between Ribosomal Protein S4 and the α Operon Messenger RNA†

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ABSTRACT: The *Escherichia coli* ribosomal protein S4 is known to repress translation of its own gene and several other ribosomal protein (r-protein) genes in the α operon as part of a general mechanism coordinating the levels of rRNA and r-protein synthesis. Using a filter binding assay and RNA transcripts prepared in vitro, we have detected and quantitated specific interactions between S4 and α mRNA fragments. The main results are the following: (i) Only the α mRNA leader is required for specific recognition, with a small fraction of the binding free energy derived from sequences at the ribosome initiation site. (ii) 16S rRNA and α mRNA compete for binding to S4 with about the same affinity ($\approx 2 \times 10^7$ M^{-1}), suggesting that S4 utilizes the same recognition features in each RNA. (iii) Nonspecific binding of S4 to tRNA or other mRNA sequences is strongly salt dependent, while the specific S4–α mRNA affinity is nearly independent of salt. (iv) At physiological salt concentrations the nonspecific S4–RNA affinity (10^5–10^6 M^{-1}) is large enough to strongly buffer the free S4 concentration in vivo.

There is considerable evidence that specific interactions between ribosomal proteins and messenger RNAs are responsible for the close coordination of ribosomal protein and ribosomal RNA synthesis in bacteria. According to the autoregulation hypothesis [reviewed by Lindahl & Zengel (1982) and Nomura et al. (1984)], certain ribosomal proteins, if accumulated in excess over ribosomal RNA, bind to specific sites on their own mRNA to repress further translation of r-proteins.[1] Each r-protein operon codes for one such r-protein repressor specific for that operon. The repressor proteins all bind directly to ribosomal RNA early in ribosome assembly and presumably use the same RNA binding site to recognize both the ribosomal and messenger RNA sites. This transla-

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[1] Abbreviations: r-protein, ribosomal protein; RBS, ribosome binding site; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; kb, kilobase; bp, base pair; EDTA, ethylenediaminetetraacetic acid.

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