Biochemistry

 Volume 25, Number 12 June 17, 1986

Accelerated Publications

Identification of the Fibroblast Growth Factor Receptor of Swiss 3T3 Cells and Mouse Skeletal Muscle Myoblasts†

Bradley B. Olwin and Stephen D. Hauschka*
Department of Biochemistry, University of Washington, Seattle, Washington 98195
Received March 4, 1986; Revised Manuscript Received April 4, 1986

ABSTRACT: Two distinct fibroblast growth factors (FGF) were purified to homogeneity from bovine brain on the basis of their ability to stimulate skeletal muscle myoblast proliferation. These growth factors are also mitogenic for Swiss 3T3 cells and appear to be closely related to or identical with previously isolated anionic and cationic fibroblast growth factors. The half-maximum concentrations (EC_{50}) for stimulation of myoblast DNA synthesis by the anionic and cationic growth factors were 30 pM and 1 pM, respectively. In contrast, an EC_{50} of 45 pM was observed for stimulation of 3T3 cell DNA synthesis by both growth factors. Binding of ^{125}I-labeled anionic FGF was saturable with apparent K_d values of 45 pM and 11 pM and approximately 60 000 and 2000 receptor sites per cell for 3T3 cells and MM14 murine myoblasts, respectively. Unlabeled anionic and cationic FGF equally displaced ^{125}I-labeled anionic FGF from 3T3 cells while cationic FGF was more potent than anionic FGF for displacement from skeletal muscle myoblasts, demonstrating that a single receptor binds the two distinct growth factors. Binding was specific for these factors since platelet-derived growth factor, insulin, insulin-like growth factor 1, epidermal growth factor, and nerve growth factor were unable to displace bound ^{125}I-labeled anionic FGF from Swiss 3T3 cells. Chemical cross-linking of specifically bound ^{125}I-labeled anionic FGF to 3T3 cells and MM14 myoblasts identified a single detergent-soluble FGF receptor with an apparent molecular weight of 165 000.

The permanent murine skeletal muscle cell line MM14 requires one or more unidentified factors from serum plus nanogram per milliliter levels of impure fibroblast growth factor (FGF)[1] to maintain proliferation (Linkhart et al., 1981). The required serum components cannot be fully replaced by supplementation with selenium, dexamethasone, serum albumin, fibronectin, transferrin, insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), nerve growth factor (NGF), or platelet-derived growth factor (PDGF), either individually or in various combinations (Hauschka, unpublished data). MM14 myoblasts are absolutely dependent on FGF for proliferation; no other known mitogen or combination of mitogens will replace FGF (Linkhart et al., 1982; Hauschka, unpublished data). In addition to its mitogenic effect for skeletal myoblasts, impure preparations of commercially available FGF specifically repress muscle cell fusion, expression of muscle-specific genes, and commitment to a postmitotic state—a differentiated phenotype in which the cells become unresponsive to further additions of FGF (Linkhart et al., 1981; Clegg, Linkhart, Olwin, and Hauschka, submitted for publication).

Purification of FGF by other investigators has demonstrated the existence of at least two forms that, on the basis of their strong affinity for heparin, have also been designated as heparin-binding growth factors (HBGF). The acid mitogens are found only in neural tissue and include acidic fibroblast growth factor (a-FGF) (Thomas et al., 1984; Gimenez-Gallego et al., 1985), class I HBGF (Lobb & Fett, 1984), endothelial cell growth factor (ECGF) (Maciag et al., 1984; Burgess et al.,

† This work was supported by grants from the NIH and MDA to S.D.H. and by an MDA postdoctoral fellowship to B.B.O.

[1] Abbreviations: HBGF, heparin-binding growth factor; a-FGF, acidic fibroblast growth factor; b-FGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; NGF, nerve growth factor; ECGF, endothelial cell growth factor; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, 0.05 M sodium phosphate, pH 7.4, and 0.10 M NaCl; DSS, disuccinimidyl suberate; Me_2SO, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography; kDa, kilodalton; DMEM, Dulbecco's modified Eagle medium.

0006-2960/86/0425-3487$01.50/0

1985), eye-derived growth factor II (Schreiber et al., 1985), and retina-derived and hypothalamus-derived growth factors (D'Amore & Klagsbrun, 1985). These factors are related by their identical affinity for heparin, isoelectric point, immunological cross-reactivity, and receptor binding cross-reactivity (Lobb & Fett, 1984; Schreiber et al., 1985). Basic fibroblast growth factors (b-FGF), which bind heparin more avidly than a-FGF, are found in a wide variety of tissues including pituitary (Bohlen et al., 1984), brain (Lobb & Fett, 1984), placenta (Gospodarowicz et al., 1985), corpus luteum (Gospodarowicz et al., 1985), macrophages (Baird et al., 1985), tumors (Shing et al., 1984), and cartilage (Sullivian & Klagsbrun, 1985). A myogenic growth factor with properties similar to those of b-FGF has recently been purified from chicken skeletal muscle tissue (Kardami et al., 1985a,b), and both classes of FGF appear to be present in extracts from 4–12-day-old chick embryos (Seed, Olwin, and Hauschka unpublished results). The basic FGF was first described by Gospodarowicz (1975); it is a potent mitogen for mesodermal and neural crest-derived cells [for a review see Gospodarowicz (1984)]. A comparison of acidic and basic FGF was first reported by Lemmon et al. (1982), and homogeneous preparations of basic FGF were first described by Lemmon and Bradshaw (1983). The complete amino acid sequences of acidic FGF (Gimenez-Gallego et al., 1985) and basic FGF (Esch et al., 1985) demonstrate that the two are distinct polypeptides with 55% sequence identity; this finding appears consistent with their similar biological activities. However, not all cells responded equivalently to the two forms of FGF, and it was unclear whether both forms of FGF are equally potent mediators for both the proliferation and repression of the differentiation of skeletal muscle myoblasts.

In this paper, we describe the purification of two fibroblast growth factors from bovine brain based on their ability to stimulate myoblast proliferation. The amino acid composition, differential affinity for heparin–Sepharose, and mitogenic activity for Swiss 3T3 cells of these two factors are consistent with their identification as the anionic and cationic fibroblast growth factors that have been isolated by other investigators (Lobb et al., 1986). Specific binding and cross-linking of ^{125}I-labeled a-FGF have identified a single class of binding sites on MM14 myoblasts and Swiss 3T3 cells that corresponds to a cell surface polypeptide receptor with an apparent molecular weight of 165 000.

Materials and Methods

Swiss 3T3 cells and PDGF were generously supplied by Russell Ross and Elaine Raines, Department of Pathology, University of Washington, NGF was supplied by John Schmidt, Department of Pharmacology, University of Washington, and EGF and insulin were supplied by Steve Pelech, Howard Hughes Medical Institute, University of Washington. IGF-1 was purchased from AmGen and heparin–Separose from Pharmacia, Inc.

Murine MM14 myoblasts (Hauschka et al., 1979; Linkhart et al., 1981) were grown on gelatin-coated culture dishes in Ham's F-10C (Ca^{2+} adjusted to 0.8 mM) supplemented with standard antibiotics, 15% horse serum, and variable concentrations of b-FGF (see below). Cultures were maintained in a 37 °C incubator at 5% CO_2 on a rocking platform (2 cycles/min) and fed at 12-h intervals with b-FGF levels adjusted from 2 to 6 ng/mL as the culture density increased from (1–2) $\times 10^5$ to a maximum of (1–1.5) $\times 10^6$ cells/100-mm dish. Swiss 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Gibco) and standard antibiotics or in the same media used for MM14 propagation. Stock cell cultures were kept at subconfluent densities by passaging twice each week.

Purification of bovine brain a-FGF and b-FGF was achieved by a combination of the procedures described by Gospodarowicz et al. (1978), Lobb and Fett (1984), and Shing et al. (1984). Purification through the CM-Sephadex column was exactly as described by Gospodarowicz et al. (1978) except that the column was washed with 0.1 M NaCl in 0.1 M NaH_2PO_4, pH 6.0, until the A_{280} reached 0 and eluted in the same buffer containing 0.6 M NaCl. The eluted protein peak was applied overnight to a 40-mL heparin–Sepharose column, which was preequilibrated in 10 mM Hepes, pH 7.4, and 0.6 M NaCl. The column was washed with the application buffer and then with the Hepes buffer containing 1.0 M NaCl until the A_{280} reached 0. a-FGF was eluted in the Hepes buffer containing 1.5 M NaCl, and b-FGF was eluted in the same buffer with 2.5 M NaCl. Both a-FGF and b-FGF pools from the first column were separately applied to and eluted from a second 10-mL heparin–Sepharose column exactly as described for the first column. This step removed minor contaminants and concentrated both growth factors. Yields were typically 0.4 and 0.1 mg/kg for a-FGF and b-FGF, respectively. Homogeneous preparations of a-FGF and b-FGF migrated at apparent molecular weights of 17 000 and 19 000, respectively. The amino acid composition of HPLC-purified a-FGF was nearly identical with those reported for acidic FGF (Thomas et al., 1984) and for class I HBGF (Lobb & Fett, 1984), while the amino acid composition of b-FGF was nearly identical with those reported for basic FGF (Bohlen et al., 1984) and for class II HBGF (Lobb & Fett, 1984). These data suggest identity of the mitogens purified by our procedures with the previously reported acidic and basic FGF from bovine brain. Protein concentrations were determined by the method of Peterson (1977) using BSA as a standard and confirmed by amino acid analysis as described by Bidlingmeyer et al. (1984).

Samples were assayed for the stimulation of MM14 DNA synthesis and the repression of differentiation by the addition of FGF-containing fractions to 24-well culture plates containing 1.0 mL of a standard medium and 500 MM14 myoblasts per well. Cultures were allowed to proliferate for 14–16 h, 3 μCi of [^{3}H]thymidine was then added to each well, and the cultures were incubated for an additional 8–10 h. The cultures were harvested by rinsing twice with PBS, precipitating labeled DNA with 3% trichloroacetic acid and 0.1% pyrophosphate for 15 min at 4 °C, rinsing twice with 70% ethanol, and solubilizing in 2% sodium dodecyl sulfate (SDS). Radioactivity was counted in 5 mL of Ecolite (Westchem) on a Beckman LS-150 scintillation counter. MM14 myoblasts exhibit an absolute requirement for FGF since G_1-phase cells deprived of FGF for as little as 3 h commit to an irreversible postmitotic state (Linkhart et al., 1981). Thus, after the initial incubation period, only wells containing sufficient levels of FGF to repress commitment and to maintain exponential growth will exhibit thymidine incorporation during the labeling period. Cultures lacking sufficient FGF levels to repress commitment to terminal differentiation exhibited multinucleated myotubes and stained positively with antisera directed against myosin heavy chain. FGF stimulation of DNA synthesis in 3T3 cells was determined by using serum-starved confluent monolayers (14–16-h exposure to DMEM containing 0.3% fetal calf serum) in 96-well culture plates. FGF in DMEM plus 3.0 μCi/mL [^{3}H]thymidine was then added to each well, and cultures were incubated an additional 24–48 h. Labeled DNA was isolated as described for MM14 cells.

Acidic FGF was radiolabeled by adding 3.5 μg (0.05 mL) of the growth factor in 10 mM Hepes, pH 7.4, and 1.5 M NaCl to 0.05 mL of Enzymobeads (Bio-Rad Laboratories), 0.025 mL of 2% glucose in H_2O, and 2.0 mCi of $Na^{125}I$. The reaction was incubated for 45 min at 23 °C. Free ^{125}I was separated from ^{125}I-a-FGF on a 2.0-mL column of Sephadex G-25 in 10 mM Hepes, pH 7.4, 0.6 M NaCl, and 0.1% BSA. Approximately 0.4 mL of ^{125}I-a-FGF was then applied to a 0.2-mL heparin–Sepharose column preequilibrated in the same buffer; the column was washed with approximately 10 mL of buffer until ^{125}I cpm reached a low level. ^{125}I-a-FGF was then eluted with the same buffer containing 2.5 M NaCl. Recovery of ^{125}I-FGF ranged from 20% to 30% with specific activities from 1.5×10^6 to 3.0×10^6 cpm/pmol of a-FGF. Electrophoresis and autoradiography of ^{125}I-a-FGF by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) revealed a single radioactive band that comigrated with unlabeled a-FGF. The biological activity of ^{125}I-a-FGF determined by the MM14 myoblast clonal growth assay was indistinguishable from that of native a-FGF (unpublished data).

Binding of ^{125}I-a-FGF and unlabeled FGF to proliferating MM14 myoblasts and to confluent monolayers of 3T3 cells was performed in 60-mm dishes and 24-well culture plates, respectively. The cells were first rinsed twice with Ham's F-10C and then once with Ham's F-10C containing 25 mM Hepes, pH 7.4, and 0.1% BSA (binding buffer). The cells were preincubated at 10 °C in binding buffer and then ^{125}I-a-FGF, and unlabeled a-FGF or b-FGF was added to initiate the binding assay; final volumes were 1.0 and 0.25 mL for MM14 myoblasts and 3T3 cells, respectively. Equilibrium was reached by incubating the cells for 3 h at 10 °C on a rotary shaker oscillating at 50 cycles/min. Binding assays were terminated by rapidly rinsing cultures 4 times with cold PBS containing 0.5% BSA, and bound ^{125}I-a-FGF was then eluted by solubilizing with PBS containing 1% Triton X-100 for 5 min and rinsing 1 time with PBS. Nonspecific binding was determined in the presence of a 100-fold molar excess of a-FGF and was subtracted from the total ^{125}I cpm to determine specifically bound ^{125}I-a-FGF. Counting was performed on a Beckman Model 4000 γ-counter.

For cross-linking FGF to its receptor, ^{125}I-a-FGF was bound to intact 3T3 cell cultures grown in 60-mm dishes as described above. Binding was terminated by rinsing 4 times with PBS; 1.0 mL of PBS was then added followed by 5 μL of 0.05 M disuccinimidyl suberate (DSS) in dimethyl sulfoxide (Me_2SO). The cells were incubated for 15 min at 4 °C, rinsed 2 times with PBS and once with PBS containing antiproteases (1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 20 units/mL aprotinin). The cells were collected by scraping in 0.5 mL of the antiprotease buffer, rinsing once with 0.5 mL of this buffer, and centrifuging at 15000*g* for 5 min. Membrane-associated proteins were extracted in 20 μL of antiprotease buffer containing 1% Triton X-100 at 4 °C for 30 min, followed by centrifugation at 15000*g* for 5 min. The Triton-soluble and -insoluble fractions were subjected to SDS–PAGE on 7.5% gels (Laemmli, 1970) and exposed to Kodak X-Omat film at −70 °C with a Du Pont Cronex Lightning Plus intensifying screen. Binding and cross-linking of ^{125}I-a-FGF to MM14 myoblasts (2×10^6 cells per 100-mm tissue culture dish) were as described for 3T3 cells above.

Results and Discussion

Homogeneous preparations of a-FGF and b-FGF were mitogenic for MM14 myoblasts (Figure 1A) and for Swiss 3T3 cells (Figure 1B). The EC_{50} values for a-FGF and b-FGF

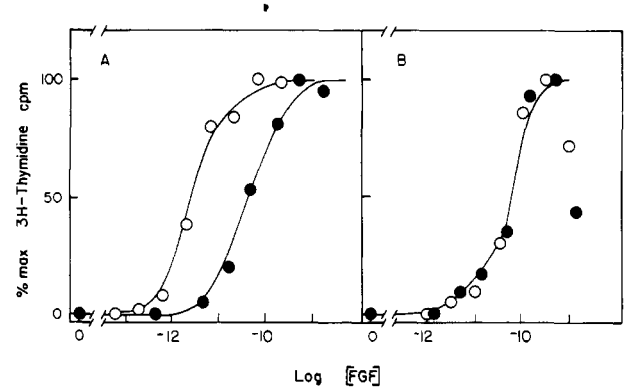

FIGURE 1: Stimulation of DNA synthesis determined by incorporation of [^{3}H]thymidine in MM14 myoblasts and Swiss 3T3 cells. (A) Data are plotted as the percent maximum [^{3}H]thymidine cpm incorporated in MM14 myoblasts by a-FGF (●) and b-FGF (○) as a function of the logarithm of the total molar concentration added. (B) The data are plotted as described in (A) for [^{3}H]thymidine incorporation in Swiss 3T3 cells by a-FGF (●) and b-FGF (○). Values for [^{3}H]-thymidine incorporation in this experiment were 366 cpm for no added FGF with 17600 and 14800 cpm at 100% stimulation of MM14 DNA synthesis for a-FGF and b-FGF, respectively. Incorporation of [^{3}H]thymidine into 3T3 cells was 7100 cpm with no added FGF with 99100 and 96520 cpm at 100% stimulation of DNA synthesis for a-FGF and b-FGF, respectively. These data are the results of a single experiment performed in duplicate. Each experiment was repeated at least 2 times with similar results.

stimulation of DNA synthesis in MM14 myoblasts were 30 ± 2 and 1 ± 0.5 pM, respectively. In contrast, the EC_{50} values of both growth factors for 3T3 cells were 45 pM in one experiment (Figure 1B) and 65 pM in a second experiment. Previous studies have shown that the mitogenic potencies of class I and II heparin-binding growth factors differ by as much as 5-fold for Balb/c 3T3 cells (Lobb & Fett, 1984). However, other investigators have found that the relative mitogenic potencies vary widely with cell type and that established cell lines usually have similar EC_{50} values for both fibroblast growth factors (Bohlen et al., 1985). Our Swiss 3T3 cell data thus concur with the latter investigations, but the MM14 myoblast data differ from the general rule of similar responsiveness for "established" cell lines since these cells have been in culture for over 1000 doublings.

Kinetic analysis of ^{125}I-a-FGF binding to confluent monolayers of Swiss 3T3 cells indicated that saturation occurred between 1 and 2 h at 10 and 37 °C (Figure 2A). Since binding of ^{125}I-a-FGF appeared constant for up to 4 h, we concluded that extensive ligand-induced receptor down-regulation of the type observed for EGF receptor (Cohen et al., 1980) does not occur in this system. This observation was confirmed by preincubating 3T3 cells with excess a-FGF for various times and observing less than 20% decreases in total cell-associated receptor numbers (Figure 2B).

Saturation and Scatchard (1949) analysis of ^{125}I-a-FGF binding to near-confluent monolayers of Swiss 3T3 cells identified a single class of noninteracting sites with a K_d of 46 ± 18 pM ($n = 3$), and calculations indicated approximately (6.0 ± 1.5) × 10^4 receptor sites per cell (Figure 3A). We have observed that the 3T3-cell FGF receptor number is highly sensitive to 3T3 cell density with a 2–3-fold increase in both receptor number per cell and K_d occurring when comparing 50% confluent cultures to confluent monolayers (unpublished data). Proliferating MM14 myoblasts exhibited 1700 and 2100 receptor sites per cell with K_d values of 8 and 13 pM for two independent experiments (data not shown).

Since the K_d for ^{125}I-a-FGF binding to MM14 myoblasts and Swiss 3T3 cells is equal to or less than the EC_{50} value for stimulation of DNA synthesis in the respective cell types, no

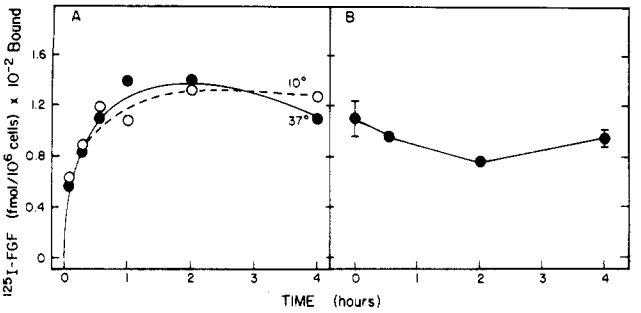

FIGURE 2: Kinetics for ^{125}I-a-FGF binding to Swiss 3T3 fibroblasts. (A) Swiss 3T3 cells were incubated with 800 pM ^{125}I-a-FGF (approximately 20 times the K_d for these cells) for the various times indicated at 37 (●) and 10 °C (○). Nonspecific binding was determined in the presence of 80 nM a-FGF and did not exceed 30% of the bound ^{125}I cpm. These data are the results of a single experiment performed in duplicate. The experiment was repeated with similar results 2 times. (B) Preincubation with a-FGF for up to 4 h does not change the FGF receptor number. Swiss 3T3 cells were preincubated at 37 °C with 630 pM a-FGF for the times indicated. An equal concentration of a-FGF was then added to the zero time point, and the specifically bound ^{125}I-a-FGF was determined for each sample as described under Materials and Methods. The ^{125}I-a-FGF bound (in femtomoles) was plotted as a function of the time the culture was preincubated with unlabeled a-FGF. A concentration of 800 pM ^{125}I-a-FGF was used to ensure saturation of binding sites. Nonspecific binding did not exceed 30% of the bound ^{125}I cpm. The data are the results of a single experiment performed in triplicate with error bars denoting the standard deviation from the mean. A second experiment yielded similar results.

"spare" receptors appear to be present in either myoblasts or 3T3 cells. This result differs from the reported presence of "spare" receptors for other 3T3 cell mitogens such as EGF (Cohen et al., 1980) as well as for ECGF, which appears to be a class I heparin-binding growth factor (Schreiber et al., 1985). It also differs from reports of "spare" receptors for L-6 myoblast growth factors such as insulin, IGF-1, and IGF-2 (Beguino et al., 1985).

Although the biological effects of a-FGF and b-FGF on 3T3 cells and MM14 myoblasts appear similar, it is unknown whether they bind to a single receptor or multiple cellular receptors. To test this hypothesis, near-confluent monolayers of Swiss 3T3 cells were incubated with a single concentration of ^{125}I-a-FGF (85 pM) in the presence of increasing concentrations of unlabeled a-FGF or b-FGF (Figure 3B). It is clear that a-FGF and b-FGF compete for a common binding site since both growth factors were equally potent for the displacement of bound ^{125}I-a-FGF. In addition, the affinities of ^{125}I-a-FGF and unlabeled a-FGF or b-FGF for binding are nearly identical since 50% of the bound ^{125}I-a-FGF is displaced by concentrations of either unlabeled FGF (100 pM) that are approximately equal to the concentration of ^{125}I-a-FGF (85 pM) present. The specificity of ^{125}I-a-FGF binding was demonstrated by the inability of 10 nM PDGF, insulin, IGF-1, EGF, or NGF to displace bound ^{125}I-a-FGF (Figure 3B). It is interesting to note the equally potent displacement of ^{125}I-a-FGF from 3T3 cells by unlabeled a-FGF and b-FGF coincided with their equivalent stimulation of 3T3 cell DNA synthesis. However, displacement of ^{125}I-a-FGF from MM14 myoblasts was achieved at significantly lower concentrations of b-FGF than a-FGF (Table I), consistent with the greater potency of b-FGF over that of a-FGF for stimulation of MM14 DNA synthesis (Figure 1A).

As an initial step in identifying and isolating the FGF receptor, ^{125}I-a-FGF was incubated with confluent monolayers of 3T3 cells, chemically cross-linked to FGF binding sites with DSS, and visualized by autoradiography of Triton X-100

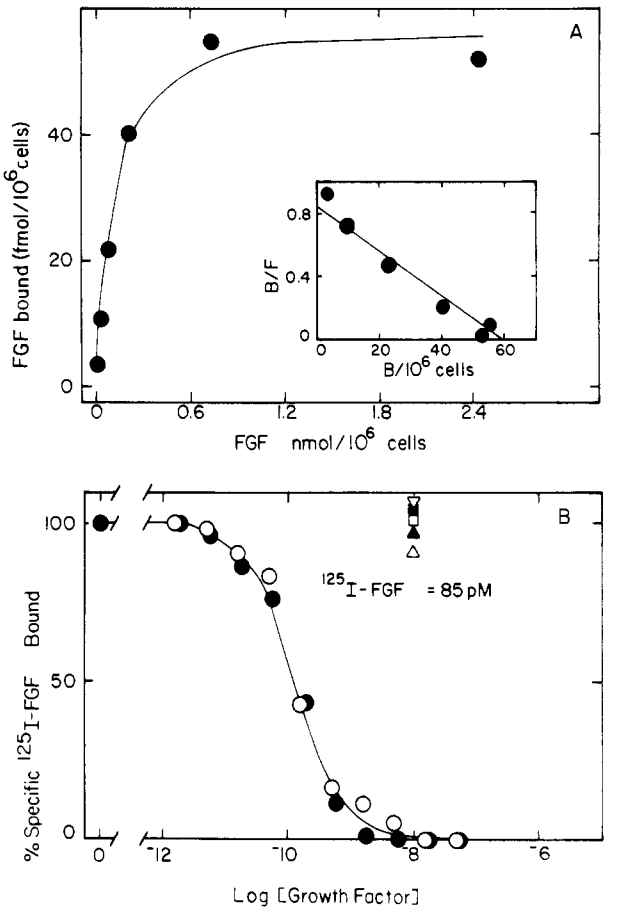

FIGURE 3: Binding of ^{125}I-a-FGF and its displacement from intact Swiss 3T3 cells. (A) Increasing concentrations of ^{125}I-a-FGF were added to 3T3 cells in the absence or presence of 0.12 μM unlabeled a-FGF at 10 °C for 3 h. The specifically bound ^{125}I-a-FGF was plotted as a function of the total ^{125}I-a-FGF added or according to Scatchard (inset). B and B/F represent femtomoles of bound ^{125}I-a-FGF per 10^6 cells and bound/free, respectively. Nonspecific binding did not exceed 30% of the total bound counts at saturation. The data plotted are the mean of duplicate points from one experiment. Two additional experiments gave similar results. (B) Displacement of ^{125}I-a-FGF by unlabeled a-FGF and b-FGF from Swiss 3T3 cells. Intact 3T3 cells were incubated with a constant concentration of ^{125}I-a-FGF (85 pM) and with increasing concentrations of unlabeled a-FGF (●) or b-FGF (○) or at 10 nM concentrations of NGF (▲), PDGF (△), insulin (▽), IGF-1 (□), and EGF (■) for 3 h at 10 °C. Nonspecific binding was determined as ^{125}I cpm that are not displaced at maximum concentrations of unlabeled FGF. Nonspecific binding did not exceed 15% of the total bound ^{125}I cpm. Specifically bound ^{125}I cpm were plotted as the percent maximum specific binding vs. the logarithm of the total added molar concentration of unlabeled a-FGF. The data are the mean of duplicate points from a single experiment. Similar results were obtained from two additional experiments, with competition occurring at 75 and 100 pM ^{125}I-a-FGF.

Table I: Displacement of ^{125}I-a-FGF from MM14 Myoblasts

	^{125}I-a-FGF displacement[a]	
	50%	100%
a-FGF	80 pM	10 nM
b-FGF	8 pM	1 nM

[a] Displacement of 60 pM ^{125}I-a-FGF by unlabeled a-FGF and b-FGF from MM14 myoblasts as described under Materials and Methods and in the legend to Figure 3B. The concentrations of unlabeled FGF given displaced 50% or 100% of the specifically bound ^{125}I-a-FGF, respectively. Nonspecific binding was approximately 20% in these experiments and was determined in the presence of 6 nM a-FGF.

solubilized membranes separated by SDS–PAGE. A single ^{125}I-labeled complex was observed migrating at an apparent molecular weight of 180000 (Figure 4A). This result suggests

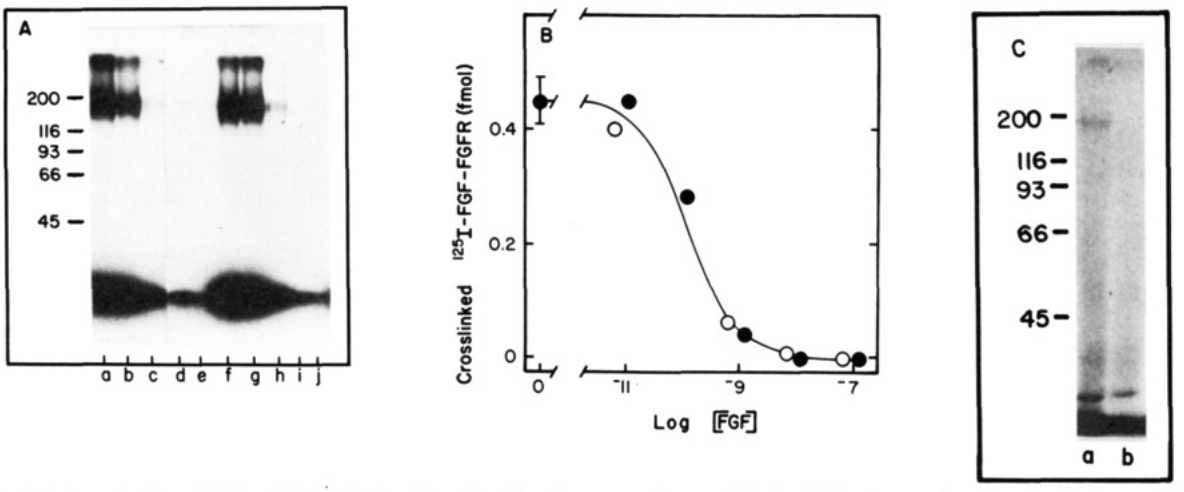

FIGURE 4: (A) Cross-linking of ^{125}I-a-FGF to Swiss 3T3 cells. Confluent monolayers of Swiss 3T3 cells were incubated with 85 pM ^{125}I-a-FGF in the presence of 0.012, 0.12, 1.2, 12, and 120 nM unlabeled a-FGF (a–e), 0.006, 0.6, 6.0 and 60 nM unlabeled b-FGF (g–j), or in the absence of unlabeled FGF (f). Triton X-100 soluble extracts were separated by 7.5% SDS–PAGE and autoradiographed at −70 °C for 5 days. Molecular weight standards were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (93 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). (No radioactive complexes were observed in the absence of cross-linking agents.) Radioactivity migrating in the low molecular weight region represents ^{125}I-FGF that was specifically bound but not cross-linked to the receptor by the DSS treatment. Radioactivity migrating in the region above 200 kDa did not enter the running gel and may represent either incompletely solubilized membranes or higher order cross-linked material. Similar results were obtained in two other experiments. (B) The ^{125}I-cross-linked complex was excised from the gel and counted. The femtomoles of ^{125}I-a-FGF covalently cross-linked to the 3T3 cell FGF receptor was plotted as a function of the logarithm of the molar a-FGF (●) and b-FGF (○) concentrations added. (C) Cross-linking of ^{125}I-a-FGF to MM14 cells. Myoblasts (2×10^6 cells per 100-mm tissue culture dish) were incubated with 150 pM ^{125}I-a-FGF (a) and ^{125}I-a-FGF plus 1.5 nM a-FGF (b), cross-linked, and processed as described for 3T3 cells. Each lane represents 2×10^6 myoblasts. The autoradiogram was exposed for 15 days at −70 °C. Similar results were obtained in an additional experiment. Molecular weight standards were identical with those described above for SDS–PAGE of 3T3 cell membranes.

an approximate molecular weight of 165 000 for the FGF receptor. Competition of a-FGF and b-FGF for ^{125}I-a-FGF binding to a single cellular site was confirmed by incubating 3T3 cells with 85 pM ^{125}I-a-FGF in the presence of increasing concentrations of unlabeled a-FGF and b-FGF, followed by cross-linking to the FGF receptor. Unlabeled a-FGF and b-FGF displaced up to 100% of the bound ^{125}I-a-FGF from the radiolabeled receptor complex with 50% inhibition occurring at concentrations similar to the concentrations of ^{125}I-a-FGF present (Figure 4A,B). The skeletal muscle FGF receptor was also identified by cross-linking ^{125}I-a-FGF to MM14 myoblasts with DSS. A single complex was observed migrating at an apparent molecular weight of 180 000 (Figure 4C). Taken together, the mitogenic responsiveness to a-FGF and b-FGF of both cell types, the specific ^{125}I-a-FGF binding, the displacement of ^{125}I-a-FGF by unlabeled a-FGF and b-FGF in 3T3 and MM14 cells, and the identification of a single cross-linked complex suggest that the 165-kDa protein is the FGF receptor responsible for mediating the biological effects of a-FGF and b-FGF. These observations suggest that a single FGF receptor on skeletal muscle myoblasts may mediate the signals for both replication and repression of differentiation. Triton X-100 insoluble samples from DSS-cross-linked cells contain no 180-kDa complex, and cross-linking of ^{125}I-a-FGF to crude 3T3 cell membranes yields an ^{125}I-labeled 180-kDa complex (data not shown), suggesting that the 165-kDa FGF-binding protein is a membrane component.

Following preparation of this paper, a report appeared describing the binding and cross-linking of basic FGF to receptors on hamster BHK-21 cells (Neufeld & Gospodarowicz, 1985). If the b-FGF used in our studies is identical with basic FGF studied by these investigators, then our results with 3T3 cells and theirs with BHK-21 cells differ in number of receptors per cell (60 000 vs. 120 000), receptor K_d (45 pM vs. 270 pM), existence of "spare" receptors (none vs. 75%), ligand-induced receptor down-regulation (≤20% vs. 70%), and number and size of FGF receptor complexes (165 kDa vs. 140 and 125 kDa). These investigators did not analyze the binding of a-FGF or the competition of a-FGF for basic ^{125}I-FGF binding sites on BHK-21 cells and suggested that the receptors for a-FGF and b-FGF are distinct. Although studies of the FGF receptor types are still preliminary, the apparent differences between the BHK-21, 3T3, and MM14 FGF receptors may be significant. These observations together with the apparent difference in mitogenic responsiveness of myoblasts to a-FGF and b-FGF raise the interesting point that FGF receptor number and FGF receptor affinity may be under different regulatory controls that are cell-type specific.

ACKNOWLEDGMENTS

We thank Harry Charbonneau for amino acid analysis, Patricia Noel for invaluable technical assistance, Russell Ross and Elaine Raines for their generous gifts of Swiss 3T3 cells, Steven Pelech, Jim Jaynes, Jean Buskin, and Elaine Raines for critical reading of the manuscript, Kate Roudybush for typing the manuscript, and Pam Horbett for the preparation of figures.

Registry No. Cationic FGF, 62031-54-3.

REFERENCES

Baird, A., Mormede, P., & Bohlen, P. (1985) *Biochem. Biophys. Res. Commun. 126*, 358–364.

Beguino, F., Kuhn, C. R., Moses, A. C., & Smith, R. J. (1985) *J. Biol. Chem. 260*, 15892–15898.

Bidlingmeyer, B. A., Cohen, S. A., & Torrin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Bohlen, P., Baird, A., Esch, F., Ling, N., & Gospodarowicz, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5364–5368.

Bohlen, P., Esch, F., Baird, A., & Gospodarowicz, D. (1985) *EMBO J. 4*, 1951–1956.

Burgess, W. H., Mehlman, T., Friesel, R., Johnson, W. V., & Maciag, T. (1985) *J. Biol. Chem. 260*, 11389–11392.

Cohen, S., Carpenter, G., & King, L., Jr. (1980) *J. Biol. Chem. 255*, 4834–4842.

D'Amore, P., & Klagsbrun, M. (1984) *J. Cell Biol. 99*, 1545–1549.
Esch, F., Baird, A., Ling, N., Veno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P., & Guillemin, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6507–6511.
Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSulvo, J., & Thomas, K. (1985) *Science (Washington, D.C.) 230*, 1385–1388.
Gospodarowicz, D. (1975) *J. Biol. Chem. 250*, 2515–2520.
Gospodarowicz, D. (1984) in *Mediators in Cell Growth and Differentiation* (Pagel, W. J., Ed.) pp 109–134, Raven, New York.
Gospodarowicz, D., Greenburg, G., Bialecki, H., & Zetter, B. (1978) *In Vitro 14*, 85–118.
Gospodarowicz, D., Cheng, J., Lui, G.-M., Fujii, D. K., Baird, A., & Bohlen, P. (1985) *Biochem. Biophys. Res. Commun. 130*, 554–562.
Hauschka, S. D., Linkhart, T. A., Clegg, C. H., & Merrill, G. F. (1979) in *Muscle Regeneration* (Mauro, A., Ed.) pp 311–322, Raven, New York.
Kardami, E., Spector, D., & Strohman, C. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8044–8047.
Kardami, E., Spector, D., & Strohman, R. (1985b) *Dev. Biol. 112*, 353–358.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lemmon, S. K., & Bradshaw, R. A. (1983) *J. Cell. Biochem. 21*, 195–208.
Lemmon, S. K. Riley, M. C., Thomas, K. A. Hoover, G. A., Maciag, T., & Bradshaw, R. A. (1982) *J. Cell Biol. 95*, 162–169.
Linkhart, T. A., Clegg, C. H., & Hauschka, S. D. (1981) *Dev. Biol. 86*, 19–30.
Linkhart, T. A., Lim, R. W., & Hauschka, S. D. (1982) *Growth of Cells in Hormonally Defined Media*, pp 867–876, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Lobb, R. R., & Fett, J. W. (1984) *Biochemistry 23*, 6295–6299.
Lobb, R. R., Sasse, J., Sullivan, R., Shing, Y., D'Amore, P., Jacobs, J., & Klagsbrun, M. (1986) *J. Biol. Chem. 261*, 1924–1928.
Maciag, T., Mehlan, T., Friesel, R., & Schreiber, A. B. (1984) *Science (Washington, D.C.) 225*, 932–935.
Neufeld, G., & Gospodarowicz, D. (1985) *J. Biol. Chem. 260*, 13860–13868.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Schreiber, A. B., Kenney, J., Kowolski, J., Thomas, K. A., Gimenez-Gallego, G., Rios-Candelore, M., DiSalvo, J., Barritault, D., Courty, J., Courtois, Y., Moenner, M., Loret, C., Burgess, W. H., Mehlman, T., Friesel, R., Johnson, W., & Maciag, T. (1985) *J. Cell Biol. 101*, 1623–1626.
Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., & Klagsbrun, M. (1984) *Science (Washington, D.C.) 223*, 1296–1299.
Sullivan, R., & Klagsbrun, M. (1985) *J. Biol. Chem. 260*, 2399–2406.
Thomas, K. A., Rios-Candelore, M., & Fitzpatrick, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 357–361.

Immunochemical Analysis of Subunit Structures of 1,4-Dihydropyridine Receptors Associated with Voltage-Dependent Ca^{2+} Channels in Skeletal, Cardiac, and Smooth Muscles†

Annie Schmid, Jacques Barhanin, Thierry Coppola, Marc Borsotto, and Michel Lazdunski*
Centre de Biochimie du CNRS, 06034 Nice Cedex, France
Received March 10, 1986; Revised Manuscript Received April 9, 1986

ABSTRACT: Previous purification studies of the 1,4-dihydropyridine receptor associated with the calcium channel of rabbit skeletal muscle had shown that it is composed of a large glycoprotein of M_r 140 000–145 000 associated with a smaller component of M_r 32 000–34 000. Specific antisera have now been prepared against the larger component (anti-140 serum) and the smaller one (anti-32 serum). The specificity of these two antisera has been analyzed by immunoblot assays with microsomal preparations of rabbit skeletal muscle. Under disulfide-reducing conditions the anti-140 serum specifically labeled a polypeptide of M_r 140 000 while the anti-32 serum labeled three polypeptides of M_r 32 000, 29 000, and 26 000. Under nonreducing conditions both the anti-140 and the anti-32 sera specifically recognized a single large polypeptide of M_r 170 000. The same type of approach showed that the dihydropyridine receptor in cardiac and smooth muscles had a polypeptide composition similar to that found in skeletal muscle with a large polypeptide of M_r 170 000–176 000 made of two different chains of about M_r 140 000 and 34 000–32 000 associated by disulfide bridges.

Ca^{2+} channels exist mainly in excitable cells (Reuter, 1983; Tsien, 1983) such as muscle and nerve but also in secretory cells such as adrenal chromaffin cells (Baker & Knight, 1984) and in some nonexcitable cells such as sperm (Kazazoglou et al., 1985). Ca^{2+} movement through voltage-dependent Ca^{2+} channels is essential in excitation–contraction coupling in cardiac and smooth muscles. Ca^{2+} channel antagonists are currently used in the treatment of angina, supraventricular tachycardia, and hypertension and are potential drugs for other related pathologies (Janis & Triggle, 1984). 1,4-Dihydropyridines (DHP)[1] (e.g., nifedipine, nitrendipine, PN200-110)

† This work was supported by the Centre National de la Recherche Scientifique, the Fondation pour les Maladies Cardiovasculaires, and the Ministère de la Recherche et de la Technologie (Grant 85.C.1137).

[1] Abbreviations: DHP, dihydropyridine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate.

0006-2960/86/0425-3492$01.50/0