The binding of biologically active, $^{125}$I-labeled basic fibroblast growth factor (FGF) to baby hamster kidney-derived cell line cells (BHK-21) was studied at 4 °C. Unlabeled FGF displaced cell surface bound $^{125}$I-FGF, but platelet-derived growth factor, epidermal growth factor, insulin, or transferrin did not. Binding was saturable both as a function of time and as a function of increasing $^{125}$I-FGF concentrations. Scatchard analysis of the binding data revealed the presence of about $1 \times 10^6$ binding sites/cell with an apparent $K_D$ of 270 pm. The number of the binding sites was down-regulated following preincubation of the cells with FGF. The density of binding sites/cell also decreased as an inverse function of cell density. When $^{125}$I-FGF binding was studied in a BHK-21 cell membrane preparation, it was found that the membranal binding site displayed a lower $K_D$ of 21 pm.

$^{125}$I-FGF was covalently cross-linked to its cell surface receptor on intact BHK-21 cells using the homobifunctional agent disuccinimidyl suberate. Two macromolecular species with an apparent molecular weight of 145,000 and 125,000, respectively, were labeled under both reducing and nonreducing conditions. Unlabeled FGF competed with $^{125}$I-FGF for binding to both macromolecular species. The labeling of the macromolecules was also inhibited by heparin. No labeling was observed in the absence of the cross-linkers or when heat-inactivated $^{125}$I-FGF was used instead of radiolabeled, biologically active FGF.

Basic fibroblast growth factor (FGF)$^3$ is a mitogen for a wide variety of mesoderm- and neuroectoderm-derived cells (1, 2). It is a basic polypeptide (pl 9.6) present under 2 forms, one of which consists of 146 amino acids (native FGF) and a truncated form that lacks the first 15 residues (des 1-15 FGF) (3). It has been isolated and characterized from brain and pituitary (4, 5), kidney (6), corpus luteum (7), adrenal gland (8), and placenta (9).

FGF, like platelet derived growth factor (PDGF) serves as a "competence factor," activating the pleiotropic response which consists of a sequential series of regulated steps including increased nutrient transport, changes in nucleotide levels, polyribosome formation, synthesis of tRNA and proteins, and eventually the induction of DNA replication and cell division (10). Exposure of cells to FGF also induces increased expression of mRNA transcripts of the onc-genes, c-my, and e-fos (11, 12). Recently, it has been demonstrated that FGF activates the phosphatidylinositol cycle, causing phosphorylation of protein kinase C and calcium mobilization in the cytoplasm of target cells (13). The relevance of these various biochemical events to the ability of FGF to trigger cell proliferation is presently unknown. By analogy to other polypeptide hormone and growth factor systems, the cellular action of FGF is presumably exerted through its interaction with specific cell surface receptors. To understand the mode of action of FGF, it is important to gain information on the structural and functional properties of these receptors. Since chemical characterization of basic FGF has shown it to be distinct from all the other known growth factors, including acidic FGF (14), one can assume that the FGF receptor is distinct from other growth factor receptors. We now report the partial characterization of the FGF receptor present in BHK-21 cells, and the identification and properties of affinity labeled cellular components which have the characteristics of a physiologically relevant receptor for FGF.

**EXPERIMENTAL PROCEDURES**

_Materials—_iodogen and DSS were from Pierce Chemical Co. Heparin-Sepharose was from Pharmacia. Na$^{109}$I was obtained from American Corp. Cellulose acetate filters (GA-8) were from Gelman. BHK-21 cells were obtained from Dr. D. Cohen (Hannah Co., Berkeley, CA). Reagents used for NaDodSO$_4$-PAGE and high and low molecular weight protein markers were from Bio-Rad. PDGF was kindly given by Dr. L. T. Williams (Howard Hughes Institute, University of California, San Francisco). Heparin, insulin, transferrin, phenylmethylsulfonyl fluoride (PMSF), dihydrotheoretol, and N-ethylmaleimide were obtained from Sigma. Bovine serum albumin (BSA) was from Schwartz-Mann. Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from Grand Island Biological Co. calf serum was obtained from Hy Clone. Tissue culture dishes were from Falcon Plastics. Tissue culture trays (24 wells) were from Costar Co. Gentamicin was from Schering Co., and fungizone from E. R. Squibb and Sons. All chemicals were of analytical grade.

_Cell Cultures—_BHK-21 cells were routinely maintained in a 1 to 1 mixture of DMEM and Ham's F-12 medium supplemented with 5% calf serum, 2 mM glutamine, 0.25 mg/ml fungizone, and 50 mg/ml gentamicin.$^2$ Adrenal cortex cells were routinely maintained in F-12 medium supplemented with 10% calf serum, 1 mM glutamine, 0.25 mg/ml fungizone, and 1 ng/ml of pituitary FGF (added every other day until culture became confluent) (4).

_Purification of Mitogens—_FGF was purified from bovine pituitary.

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$^1$ The abbreviations used are: FGF, basic fibroblast growth factor; BHK-21, baby hamster kidney-derived cell line; NaDodSO$_4$-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's modified Eagle medium; DF medium, a 1 to 1 (v/v) mixture of DMEM and Ham's F-12 medium; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; PBS, 0.01 M Na phosphate, pH 7.4, 0.9% NaCl; DSS, disuccinimidyl suberate; $\beta$TGF, $\beta$-transforming growth factor.

Characterization of the FGF Receptor

kidney, adrenal gland, and corpus luteum, as previously described (4–8). Epidermal growth factor was purified from mouse submaxillary glands as described by Savage and Cohen (16).

Iodination of FGF—iodogen was dissolved in CHCl₂ to a concentration of 10 μg/ml. 100 μl were added to a glass tube (12 × 75 mm) and evaporated under a stream of nitrogen. Phthaloyl FGF (3.3 μg in 50 μl of 10 mM Tris-HCl, pH 7.1, and 2 μl NaCl) together with 60 μl of 0.2 mM sodium phosphate, pH 7.2, was added to the tube. The reaction was started by the addition of a 2-fold molar excess of Na₂¹²⁵I. After 15 min at room temperature, the reaction was stopped and eluted from the column with 1.35 ml of an elution buffer containing 20 mM sodium phosphate, pH 7.2, and 0.6 M NaCl (wash buffer). The reaction mixture was applied on a heparin-Sepharose column equilibrated in 20 mM sodium phosphate buffer, pH 7.2, and 2 M NaCl. The specific activity of '*I-FGF was usually 2 × 10⁶ cpm/ng FGF. The overall recovery of '*I-FGF following heparin-Sepharose affinity chromatography purification was about 20%. This was determined by going through the iodination procedure in the absence of Na₂¹²⁵I and iodo- gen. The FGF that was recovered at the end of the experiment was assayed for biological activity and compared with the activity of native FGF from the same batch. To the sample of '*I-FGF was frozen immediately and then stored at −70°C. Alternatively, 150 μl of 2% gelatin in elution buffer were added to it (final concentration 0.2% gelatin), and the mixture was kept for up to 3 weeks at 4°C. Since '*I-FGF has a high affinity for BSA, under no conditions should it be substituted for gelatin. The iodinated material was reconstituted for use in structural and biological experiments by NaDodSO₄/PAGE and for biological activity as described below. '*I-FGF was stable for 1 month after iodination.

NadodSO₄-PAGE—Aliquots of '*I-FGF cell extract, or growth medium containing '*I-FGF were added to a buffer containing 15% glycerol (v/w), 2% NaDodSO₄ (w/v), 75 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.1 mM PMSF, 0.5% ethanol (v/v), and 1 mM Z-mercaptoethanol; 0.05% bromphenol blue, and 0.15 M dithiothreitol (17). The samples were boiled for 5 min and then analyzed on an exponential gradient (10–18%) or straight 7% polyacrylamide NaDodSO₄ slab gel with a 3% stacking gel. Samples were run reduced. In some experiments nonreduced samples which did not receive dithiothreitol and were not boiled were analyzed.

After electrophoresis, the gels were stained with 0.1% Coomassie Blue in 50% trichloroacetic acid for 15 min and destained overnight with 7% acetic acid. The gels were then dried and subjected to autoradiography at −70°C using X-OMat Kodak AR film and DuPont Lightning Plus intensifying screens. High and low molecular weight protein markers were used to determine the molecular weight of the samples.

Biological Activity of '*I-Labeled and Native FGF—2 × 10⁶ adrenal cortex cells were seeded in 35-mm culture dishes in F-12 medium supplemented with 10% calf serum and antibiotics. 10-μl aliquots of '*I-labeled or native FGF diluted to the appropriate concentrations in DMEM/0.5% BSA were added every other day at the final concentrations indicated in the figure legends. After 6 days in culture, the cells were trypsinized and counted in a Coulter counter as previously described (4).

Trichloroacetic Acid Precipitation of '*I-FGF—Aliquots of '*I-FGF containing medium were added to 0.5 ml of PBS containing 1 mg of BSA, and 200 μl of 100% trichloroacetic acid were added. An aliquot was then counted. After a 15-min incubation on ice, the suspension was centrifuged for 30 s in a microcentrifuge. An aliquot of the supernatant was taken for counting and the pellet was redissolved in 0.2 ml of 0.1 N NaOH. Volume was adjusted to 0.8 ml with the addition of 0.6 ml of water and proteins were precipitated by adding 200 μl of 100% trichloroacetic acid. The final pellet was then counted in a Beckman model 5500 γ-counter.

Binding of '*I-FGF to BHK-21 Cells—BHK-21 cells were grown to confluence in 10-cm dishes (3.3 × 10⁶ cells/dish). Confluent cultures were transferred to 4°C and all subsequent operations were done in the cold. The cells were washed once with 500 μl of cold PBS and 200 μl of a binding buffer composed of F-12 medium, 25 mM HEPES, pH 7.5, and 0.2% gelatin were added to each of the wells. '*I-FGF was then added to the wells to the desired concentration. Non-specific binding was usually determined in the presence of an excess (2 μg) of pure unlabeled pituitary FGF. Both total and non-specific binding were determined in duplicated wells.

The cells were incubated for 4 h in the cold. At the end of the incubation, they were washed three times with 0.5 ml of PBS supplemented with 0.1% BSA. Subsequently, each well received 0.5 ml of 1% Triton X-100 in water and 0.1% BSA. After 1 min, an aliquot was taken and the radioactivity determined in a Beckman model 5500 γ-counter. Non-specific binding to the cells, as determined in the presence of an excess (2 μg) of pure native pituitary FGF, was usually about 20% or 1% of the total binding. At saturating concentrations of '*I-FGF, the non-specific adsorption of '*I-FGF to the dishes did not exceed 5% of the total binding.

All the experimental measurements were run in duplicate or triplicate, and all experiments were done at least twice.

Membrane Isolation—BHK-21 cells were grown to confluence in 10 15-cm dishes as described above. All the subsequent steps were done at 4°C. Each dish was washed with 10 ml of cold PBS, 7 ml of buffer A containing 20 mM MOPS, pH 7.5, 0.25 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 0.5% ethanol (v/v), and 2 mM-mercapto- ethanol were added to each dish and the cells were scrapped off with a rubber policeman. The resulting cell suspension was homogenized in a Potter-Elvehjem homogenizer at high speed with 10 strokes of a tight fitting pestle. This homogenate was centrifuged at 2,000 × g for 10 min. The resulting pellet was rehomogenized in 7 ml of buffer A and centrifuged at 2,000 × g for 10 min. The 2,000 × g supernatants were centrifuged at 100,000 × g for 60 min (20°C). The resulting pellet was resuspended in 3.3 ml of buffer B which differed from buffer A by having a sucrose concentration of 0.45 M. The membranes were frozen and stored in liquid nitrogen. About 20 to 30% of the binding sites for '*I-FGF were usually lost during the freezing process. Protein was determined by the Lowry method (18), according to the modifications of Peterson et al. (17). The final membrane protein concentration was 1 to 5 mg/ml.

Binding of '*I-FGF to Membranes—10 to 20 μg of membrane protein were used per binding assay. The final volume was 80 μl. The binding buffer contained 20 mM MOPS, pH 7.5, 2 mM MgCl₂, 140 mM NaCl, and 0.2% gelatin (w/v). '*I-FGF was added to a concentration of 50,000 cpm/ng FGF. The overall binding of '*I-FGF to the membranes was determined, unless otherwise stated, in the presence of 1 μg/ml of native pituitary FGF and the binding was done at 24°C. Binding was started by adding the crude membrane preparation to the system. After 30 min, 1 ml of cold wash buffer identical in composition to the binding buffer except that it contained 0.2% BSA instead of gelatin was added. The membranes were washed four times with 10 ml of cold PBS. The filters were then counted in a Beckman model 5500 γ-counter. Nonspecific binding of '*I-FGF to the dishes, as determined in the presence of an excess of '*I-FGF, was usually about 20% or 1% of the total binding.
and subsequently 0.7 ml of cold PBS containing 1 mM PMSF and 1 mM EDTA were added to each dish. The cells were scraped off with a rubber policeman and the cell suspension was centrifuged for 30 s in a microcentrifuge. The supernatant was aspirated and the pellet was solubilized in cold lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.1 mM EDTA, and 1 mM PMSF) for 10 min. The suspension was centrifuged as before and aliquots from the supernatant were taken for analysis by NaDodSO4-PAGE.

Heat Inactivation of FGF—125I-FGF was incubated for 5 min at 70 °C. This treatment inactivates the biological activity of native FGF by more than 95%.

RESULTS

Structural and Biological Integrity of Radioiodinated FGF—The preparations of bovine pituitary FGF used in these studies contain one major 16-kDa polypeptide as judged by NaDodSO4-PAGE, and silver staining of the gels (4, 5, 21). Pituitary FGF was iodinated to a high specific activity by the iodogen method as described under "Experimental Procedures" and 125I-FGF was separated from free Na125I by affinity chromatography on heparin-Sepharose. The content of free 125I in the final preparation was less than 0.5% as assessed by trichloroacetic acid precipitation. When the radiolabeled FGF was submitted to NaDodSO4-PAGE analysis on an exponential 10 to 18% gel gradient (4), a single radiolabeled band of about 16 kDa was detected by autoradiography (Fig. 1, inset). Thus 125I-FGF has structural properties similar to those of native FGF. Radiolabeling of polypeptide hormones has been reported as potentially damaging for their biological activity (22). We have therefore compared the biological activity of native and 125I-FGF in inducing adrenal cortex cell proliferation. As shown in Fig. 1, the potency of 125I-FGF was very close to that of native FGF. Similar results were obtained when brain cortex-derived capillary endothelial cells were used as target cells.

Characteristics of Binding of 125I-FGF to BHK-21 Cells—In order to characterize the FGF binding site successfully, a cell line with a high density of FGF receptors was required. The BHK-21 cell line was chosen because the concentration of FGF needed to stimulate cell growth was 10 to 100-fold higher than in other cell types (2, 4), indicating that this cell type could possess a high density of FGF receptors/cell. In order to minimize possible internalization of 125I-FGF, binding studies as a function of concentration and time were done at 4 °C. As shown in Fig. 2, when binding was analyzed as a function of 125I-FGF concentration, apparent saturation of binding was achieved at 22 ng/ml. Nonspecific FGF binding at 22 ng/ml averaged 18 to 20% in separate experiments. Scatchard analysis of these data is shown in the inset of Fig. 2. A straight line was obtained by fitting the data using a linear least square program. The Scatchard plot indicates that a single class of binding sites is most likely over the concentration range of FGF studied. The apparent dissociation constant for the interaction of FGF and BHK-21 cells was found to be 2.7 × 10−10 M. Approximately 120,000 binding sites are found per cell.

The time course of association of FGF to BHK-21 cells was investigated at 4 °C (Fig. 3). Apparent equilibrium was reached after 2 h. Binding was reversible since the amount of specifically bound 125I-FGF decreased by 50% when the cells were rinsed with PBS and incubated at 4 °C with fresh binding buffer for 2 h (data not shown).

Specificity of Binding—125I-FGF binding showed a high degree of specificity. Neither insulin (5 μg/ml), epidermal growth factor (2 ng/ml), PDGF (1 μg/ml), or transferrin (50 μg/ml) were able to displace efficiently the specific binding of 125I-FGF either in a cell membrane binding assay (Fig. 4), or in whole cells (Table I). In contrast, FGF purified from kidney (21), adrenal gland (8), or corpus luteum (7) was as effective as pituitary FGF in displacing 125I-FGF from its cell surface receptor (Table I). FGF purified from adrenal gland was in fact more effective in displacing 125I-FGF than pituitary-derived FGF (Table I).

![Fig. 1. A, structural and biological activity of 125I-FGF: induction of adrenal cortex cell proliferation by native pituitary FGF and 125I-FGF. Adrenal cortex cells were plated in P-12 medium containing 10% calf serum and the indicated concentration of 125I-FGF and native FGF. Mitogens were added every other day. After 6 days in culture, cells were trypsinized and counted. The concentration of 125I-FGF was calculated, assuming an overall recovery of 20% from the heparin-Sepharose column used in the iodination. The recovery from the column was determined as described under "Experimental Procedures" in a separate experiment using native FGF. B, NaDodSO4-PAGE analysis of 125I-FGF; samples containing 275 pg of 125I-FGF (55,000 cpm) were electrophoresed on a 10% to 18% exponential polyacrylamide gel. Electrophoresis was performed for 4 h at 20 mA. The migration of the samples was compared to that of protein standards, which include phosphorylase b (M, = 92,500), BSA (M, = 66,000), ovalbumin (M, = 45,000), carboxic anhydrase (M, = 31,000), soybean trypsin inhibitor (M, = 21,500), and lysozyme (M, = 14,400). An autoradiogram (48 h) from the fixed and dried gel is shown. A similar migration pattern was observed regardless of whether or not the samples were run under reducing conditions.](image1)

![Fig. 2. Concentration dependence of 125I-FGF binding to BHK-21 cells. Various concentrations of 125I-FGF were incubated with confluent BHK-21 cells at 4 °C for 4 h. The binding was performed as described under "Experimental Procedures." Nonspecific binding was determined in the presence of 3 μg/ml of unlabeled pituitary FGF. The amount of 125I-FGF specifically bound was then determined. The data plotted according to Scatchard (47) is shown in the inset. Nonspecific binding was 20% of the total binding when the concentration of 125I-FGF was 50 ng/ml. The M, = 16,000 of FGF was used to calculate Kd and the receptor number.](image2)
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FIG. 3. Time course of \(^{125}\text{I}-\text{FGF}\) binding to BHK-21 cells at 4 °C. Confluent BHK-21 cells were incubated with 200 μl of binding buffer containing 25 ng/ml of \(^{125}\text{I}-\text{FGF}\). The cultures were incubated for the indicated time intervals, washed, and measured for cell-associated \(^{125}\text{I}-\text{FGF}\) as described under “Experimental Procedures.” Nonspecific binding was determined in the presence of 2 pg/ml of unlabeled pituitary FGF. The total cell-associated binding was then corrected for nonspecific binding.

FIG. 4. Effect of EGF and PDGF on the binding of \(^{125}\text{I}-\text{FGF}\) to BHK-21 cell membranes. BHK-21 membranes (18 μg of membrane protein) were incubated for 30 min at 24 °C in the presence of 22 ng/ml of \(^{125}\text{I}-\text{FGF}\) and various concentrations of epidermal growth factor (O), PDGF (□), or FGF (●). The binding was performed as described under “Experimental Procedures.” 12 ng of \(^{125}\text{I}-\text{FGF}\) were bound to 1 mg of membrane protein when the binding was done in the absence of unlabeled ligands (100%). Data are the average of duplicate determinations. The nonspecific adsorption of \(^{125}\text{I}-\text{FGF}\) to the filters was subtracted as described under “Experimental Procedures.”

When \(^{125}\text{I}-\text{FGF}\) was displaced from BHK-21 cells by increasing amounts of native pituitary FGF, the displacement curve (Fig. 5) did not allow a clear distinction between high affinity and low affinity binding sites for \(^{125}\text{I}-\text{FGF}\). If the affinity of the labeled agonist remains unchanged, at a concentration of unlabeled competing FGF equal to the concentration of labeled FGF one would expect that a 50% displacement of the \(^{125}\text{I}-\text{FGF}\) from high affinity sites should occur. This was clearly not the case since less than 10% of the \(^{125}\text{I}-\text{FGF}\) that bound to the cells was displaced when cells were incubated in the presence of unlabeled FGF at a concentration equal to the concentration of the \(^{125}\text{I}-\text{FGF}\).

Modulation of FGF Receptor Density on BHK-21 Cells as a Function of Pre-exposure to FGF and as a Function of Cell Density—BHK-21 cells maintained in serum-supplemented medium were exposed or not to 50 ng/ml of native FGF for 3.5 h at 37 °C. To control for nonspecific unlabeled FGF binding to the cells, as well as for possible unlabeled FGF carried over in the binding assay, FGF was added at the end of the incubation, to control cultures which were previously not exposed to it. The cultures were transferred to 4 °C, washed three times to remove excess native FGF, and then exposed to \(^{125}\text{I}-\text{FGF}\) as described in Table II. The amount of receptors/cell decreased by about 75% in cultures that were pre-exposed for 3.5 h to saturating concentrations of FGF. This decrease was not caused by the presence of unlabeled FGF carried over from the preincubation, since, as mentioned above, all cultures were exposed, although for shorter periods of time for control cultures, to the same amounts of unlabeled FGF before the binding.

The relationship between the number of FGF receptors/cell and cell density was addressed in the experiment shown in Fig. 6. The number of FGF receptors/BHK-21 cell decreased almost 3-fold as cell density increased from \(6 \times 10^5\) to \(10^6\) cells/ml.

### TABLE I

<table>
<thead>
<tr>
<th>Competing ligand</th>
<th>Concentration (μg/ml)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary FGF</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Kidney FGF</td>
<td>1.0</td>
<td>105</td>
</tr>
<tr>
<td>Adrenal cortex FGF</td>
<td>1.0</td>
<td>158</td>
</tr>
<tr>
<td>Corpus luteum FGF</td>
<td>1.0</td>
<td>114</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
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</tr>
<tr>
<td>Insulin</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>Transferrin</td>
<td>50.0</td>
<td>0</td>
</tr>
</tbody>
</table>

The displacement of \(^{125}\text{I}-\text{FGF}\) produced by the other competing agents was compared to this value.

FIG. 5. Displacement of cell-associated \(^{125}\text{I}-\text{FGF}\) by increasing concentrations of unlabeled pituitary FGF. Confluent BHK-21 cell cultures were incubated with 28 ng/ml of \(^{125}\text{I}-\text{FGF}\) and increasing concentrations of unlabeled FGF for 4 h at 4 °C. The binding was performed as described under “Experimental Procedures.” When the binding was done in the absence of competing FGF, 1.5 ng of \(^{125}\text{I}-\text{FGF}\) were bound to 8 × 10⁵ cells (100%).
TABLE II
Down regulation of FGF receptors present on BHK-21 cells

BHK-21 cells were grown to confluence in 16-mm Costar wells in the presence of serum supplemented medium as described under "Experimental Procedures." The growth medium was removed, and 0.2 ml of fresh growth medium were added to each well. Unlabeled FGF was then added to the wells at the final concentrations given in the table. The cells were incubated for 3.5 h at 37 °C, and at the end of the incubation period, unlabeled FGF was added to a final concentration of 50 ng/ml to the wells that had not received it previously. The cells were then transferred to 4 °C, each well was washed with three 1-ml portions of cold PBS, and 125I-FGF was subsequently bound to the cells as described under "Experimental Procedures." The concentration of 125I-FGF was 25.2 ng/ml, and the maximal specific binding (100%) was 915 pg of bound/well.

<table>
<thead>
<tr>
<th>FGF concentration (ng/ml)</th>
<th>125I-FGF specifically bound</th>
<th>% of maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>50</td>
<td>34</td>
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</table>

FIG. 6. The effect of the cell density on the number of FGF receptors present on the cell surface of BHK-21 cells. BHK-21 cells were seeded at densities of 5, 10, 20, 40, and 100 x 10^4 cells in 16-mm wells. The cells were grown in DF medium supplemented with 5% calf serum until the cultures containing the highest initial cell concentration reached confluency (60 h). The number of cells in each of the wells was then determined as described under "Experimental Procedures." The cultures were washed with PBS and 125I-FGF (23 ng/ml) was bound to the cells. The cell-associated 125I-FGF was determined as described under "Experimental Procedures." Nonspecific binding was determined in the presence of 2 μg/ml of unlabeled pituitary FGF. The M₀ = 16,000 of FGF was used to calculate cell receptor densities.

to 7 x 10⁴ cells/dish. This result demonstrates that some feedback mechanism regulates receptor density as a function of cell density and growth stage.

Degradation of 125I-FGF by BHK-21 Cells—Confluent BHK-21 cells were incubated at 37 °C for 6 h with 125I-FGF in the presence or absence of an excess of native FGF. At the end of the incubation, an aliquot of the medium was analyzed by NaDodSO₄-PAGE. After autoradiography, the gel was cut, and each segment was counted in a γ-counter (Fig. 7). No differences in radioactivity distribution were observed between cultures which were incubated with 125I-FGF alone and those incubated with 125I-FGF plus an excess of unlabeled FGF. The distribution of radioactivity in the gel was identical to that observed when aliquots of 125I-FGF and growth medium were incubated under similar conditions in the absence of BHK-21 cells (data not shown). When the growth medium was analyzed by trichloroacetic acid precipitation, the amount (20%) of nonprecipitable radioactivity found in the growth medium of cultures exposed to 125I-FGF alone for 6 h versus cultures exposed to 125I-FGF and an excess of unlabeled FGF was identical.

Binding of 125I-FGF to BHK-21 Cell Membranes—The binding of 125I-FGF to a crude BHK-21 cell membrane preparation as a function of 125I-FGF concentration is shown in Fig. 8. Apparent saturation of binding was achieved at 20 ng/ml. A Scatchard analysis of these data shows the existence of both high and low affinity binding sites. The apparent dissociation constant of the high affinity binding sites was 2.1 x 10⁻¹¹ M. This affinity is 13-fold higher than the affinity of 125I-FGF for its receptor in whole cells (Fig. 2). Similar results were obtained when the crude membrane preparations were further purified by discontinuous sucrose gradient centrifugation.

When 125I-FGF was displaced from its membrane binding sites by increasing concentrations of unlabeled FGF (Fig. 9), the concentration of unlabeled FGF required to displace 50% of bound 125I-FGF was equal to that present in the binding medium. The binding of 125I-FGF to BHK-21 cell membranes could not be inhibited by treating the membranes with either
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**Fig. 8.** Concentration dependence of $^{125}$I-FGF binding to BHK-21 cell membranes. BHK-21 cell membranes (20 μg) were incubated for 30 min, at 24°C in the presence of the indicated concentrations of $^{125}$I-FGF. The binding was performed as described under "Experimental Procedures." Nonspecific binding was determined in the presence of 1 μg/ml of unlabeled pituitary FGF. The amount of $^{125}$I-FGF specifically bound was then determined. Data are the average of duplicate determinations, and were corrected for nonspecific binding. The amount of $^{125}$I-FGF specifically bound was 20% of the total binding when the concentration of $^{125}$I-FGF was 50 ng/ml.

**Fig. 9.** Competitive inhibition of the binding of $^{125}$I-FGF to BHK-21 cell membranes by unlabeled pituitary FGF. BHK-21 cell membranes (20 μg) were incubated for 30 min at 24°C in the presence of 14 ng/ml of $^{125}$I-FGF plus the concentrations of unlabeled FGF indicated in the figure. The binding was performed as described under "Experimental Procedures." Nonspecific adsorption of $^{125}$I-FGF to the filters was determined as described under "Experimental Procedures." This value was subtracted from the results shown in the figure. The data plotted according to Scatchard (47) is shown in the inset. Nonspecific binding was 20%; of the total binding when the concentration of $^{125}$I-FGF was 50 ng/ml.

**Fig. 10.** Affinity labeling of intact BHK-21 cells with $^{125}$I-FGF. Confluent cultures of BHK-21 cells (5 × 10⁶ cells/5-cm culture dish), were incubated with 20 ng/ml of $^{125}$I-FGF (lanes A to D), or with 10.5 ng/ml of $^{125}$I-FGF (lanes E to I). Cultures received, in addition, the following concentrations of unlabeled FGF: 4 ng/ml (lane F), 7 ng/ml (lane G), 30 ng/ml (lane H), and 200 ng/ml (lane I). Cultures were incubated at 4°C for 2.5 h. They were then washed and cross-linked to bound $^{125}$I-FGF with 0.15 mM DSS. After cross-linking, cells were scraped off the dishes, sedimented, and extracted with 0.5% Nonidet P-40. Aliquots were subjected to NaDodSO₄-PAGE on 0.75-mm thick 7% gels and autoradiography. The gel extracts shown in lanes B and D were not reduced prior to electrophoresis. The rest of the samples were reduced prior to electrophoresis. The resulting autoradiograms (5 days exposure) are shown. The molecular size markers used were myosin ($M_s = 200,000$), β-galactosidase ($M_s = 116,000$), phosphorylase b ($M_s = 92,000$), BSA ($M_s = 66,000$), ovalbumin ($M_s = 45,000$), and carbonic anhydrase ($M_s = 31,000$).

-FGF CONCENTRATION (ng/ml)

**Fig. X.** Concentration dependence of $^{125}$I-FGF binding to RHK-21 cell membranes. RHK-21 cell membranes (20 pg) were incubated for 30 min, at 24°C in the presence of the indicated concentrations of $^{125}$I-FGF. The binding was performed as described under "Experimental Procedures." Nonspecific binding was determined in the presence of 1 pg/ml of unlabeled pituitary FGF. The amount of $^{125}$I-FGF specifically bound was then determined. Data are the average of duplicate determinations, and were corrected for nonspecific binding. The amount of $^{125}$I-FGF specifically bound was 20% of the total binding when the concentration of $^{125}$I-FGF was 50 ng/ml.

**Fig. I.** Competitive inhibition of the binding of $^{125}$I-FGF to RHK-21 cell membranes by unlabeled pituitary FGF. RHK-21 cell membranes (20 pg) were incubated for 30 min at 24°C in the presence of 11 ng/ml of $^{125}$I-FGF plus the concentrations of unlabeled FGF indicated in the figure. The binding was performed as described under "Experimental Procedures." When the binding was done in the absence of competing FGF, 5 ng of $^{125}$I-FGF were bound to 1 mg of membrane protein (100%). Data are the average of duplicate determinations. The nonspecific adsorption of $^{125}$I-FGF to the filters was determined as described under "Experimental Procedures." This value was subtracted from the results shown in the figure.

-N-ethylmaleimide (10 mM, 10 min at 25°C), or dithiothreitol (10 mM, 10 min at 25°C).

Affinity Cross-linking of $^{125}$I-FGF to Its Receptor—The membrane binding sites in intact BHK-21 cell preparations were further characterized by affinity cross-linking techniques. In these experiments, $^{125}$I-FGF specifically bound to cells was covalently linked to its binding sites by using the homobifunctional cross-linker DSS. Analysis by NaDodSO₄-PAGE under nonreducing conditions revealed two macromolecular species representing the products of the cross-linking reaction (Fig. 10). The molecular weight of these species was 160,000 and 140,000. Assuming that these weights include that of pituitary FGF, then the corresponding labeled native molecules prior to cross-linking would have $M_s = 145,000$ and 125,000.

A similar distribution between the high and low molecular weight form of the receptor was observed when the gels were run following reduction of the cross-linked products (lanes A to D, Fig. 10). This indicates that they are not part of a higher molecular weight complex. No cross-linking of $^{125}$I-FGF to a higher molecular weight complex was observed, when the experiment was performed either in the absence of cells, or without DSS, or with heat-inactivated $^{125}$I-FGF (data not shown).

The cross-linking of $^{125}$I-FGF to both macromolecular species was inhibited as a function of the concentrations of unlabeled FGF (Fig. 10, lanes E to I). This inhibition of cross-linked $^{125}$I-FGF by unlabeled FGF is consistent with the inhibition of specific membrane binding assessed by filtration assays, and unlabeled FGF displaces equally well radiolabeled FGF from both $M_s = 145,000$ and 125,000 cross-linked species. The labeling of these macromolecules could also be prevented by 0.5 mg/ml of heparin (Fig. 11, lane F). Both the $M_s = 145,000$ and 125,000 cross-linked species had a high affinity for purified FGF since they could be labeled using $^{125}$I-FGF at concentrations below the $K_s$ level (Fig. 11, lane C). It also indicates that the affinity of both macromolecular species for FGF is approximately the same.
Characterization of the FGF Receptor

Kidney FGF differs from pituitary FGF in that it lacks the first 15 amino acids of the N-terminal (6, 21). However, when it was iodinated and cross-linked to the BHK-21 cells, the same two macromolecules were labeled (Fig. 11, lane E), indicating that the amino-terminal does not play a role in the interaction of FGF with its cell surface receptor.

**DISCUSSION**

This report demonstrates the presence of high affinity FGF receptor sites in BHK-21 cells. This cell line was chosen because the concentrations of FGF that are needed for induction of proliferation are 10-fold higher than in other normal diploid cells (2, 4). It therefore seemed reasonable to assume that BHK-21 cells would contain a larger number of FGF receptors/cell. FGF was iodinated to a high specific activity, and, in spite of this, retained its full biological activity. The binding of 125I-FGF to its receptor was time- and concentration-dependent, saturable, reversible, and competed for by FGF isolated either from brain, pituitary, kidney, corpus luteum, or adrenal gland. The binding was observed in the cold, suggesting a cell surface location for this site. Supporting this conclusion is the ability of 125I-FGF to bind to a crude membrane mixture prepared from BHK-21 cells.

The binding seems to be very specific to FGF, since EGF, PDGF, insulin, or transferrin do not displace 125I-FGF. In contrast, native FGF isolated from various sources (brain, pituitary, kidney, corpus luteum, adrenal gland) compete with and displace bound pituitary FGF. Since both kidney- and corpus luteum-derived FGF are a truncated form of pituitary FGF, missing the first 15 residues of the amino-terminal portion of pituitary FGF, it indicates that this portion of the molecule is not part of the FGF binding domain. This result was not unexpected since in other studies it has been shown that native or des-1–15-FGF are as potent in stimulating the proliferation of various types of mesoderm-derived cells (2, 7).

When the binding of 125I-FGF to BHK-21 cells as a function of 125I-FGF concentration is studied, a single class of high affinity binding sites is found. The binding sites have a dissociation constant of 270 pM, which is about 4-fold higher than the ED50 for FGF in this cell type. This could mean that only part of the receptor population needs to be occupied in order to get the full biological response to FGF in this cell type. It is therefore possible that a spare number of receptors exist for FGF, as has been proposed for other polypeptide hormone receptor systems, such as the insulin receptor (23, 24), the βTGF receptor (25), or the EGF receptor (26). When the ability of native FGF to displace 125I-FGF from BHK-21 binding sites was analyzed, we observed no clear distinction between displacement from high affinity versus other, lower affinity sites. In addition, it was found that the concentration of unlabeled FGF needed to displace 50% of the bound 125I-FGF was about 10-fold higher than the concentration of 125I-FGF present in the binding system (as measured by its biological activity). Since Scatchard analysis of the binding data (Fig. 2) reveals only the presence of one class of high affinity binding sites present on the cell surface, it could indicate that the affinity of 125I-FGF to its receptor on BHK-21 cells is higher than that of native FGF. Similar observations were previously made in the case of binding of 125I-PDF to human fibroblasts (27).

125I-FGF does not seem to be rapidly degraded by BHK-21 cells maintained in the presence of 5% calf serum, since no degradation products of 125I-FGF could be detected in the growth medium of BHK-21 cells, either by trichloroacetic acid precipitation or by analyzing the growth medium by NaDSSy-PAGE. It must, however, be kept in mind that BHK-21 cells grown in the presence of 5% serum are not dependent on FGF in order to proliferate. It is only when cells are exposed to defined medium supplemented with high density lipoproteins and transferrin that a requirement for FGF becomes stringent. This might explain the lack of FGF degradation by cells grown in serum-supplemented medium.

When the binding of 125I-FGF to cell membranes prepared from BHK-21 cells was characterized, it was found that the concentration of native FGF that was needed to displace 50% of the bound 125I-FGF was equal to the concentration of 125I-FGF as determined by biological activity tests. This result is in contrast with the result that was obtained when similar determinations were done in whole cells. Scatchard plot analysis of the binding of 125I-FGF to BHK-21 cell membranes also revealed that the dissociation constant of 125I-FGF is 21 pM. This is 13-fold lower than the dissociation constant found in whole cells, and 3-fold lower than the ED50 for FGF in this cell type. The reason for this affinity shift remains unclear but could perhaps be due to the membrane preparation procedure.

In order to identify the binding site present on the BHK-21 cell surface, 125I-FGF was cross-linked covalently to its receptor using DSS. This methodology has been useful in characterizing receptors for insulin (28, 29), insulin-like growth factors I and II (30–32), α- and β-TGFs (25, 33, 34), platelet-derived growth factor (35), and other polypeptide hormones (36–38). Two macromolecular species are specifically labeled by 125I-pituitary-FGF, with respective molecular weights of 145,000 and 125,000. A similar labeling pattern was obtained when 125I-kidney-derived FGF was used. The cross-linking of 125I-FGF to both species was inhibited by heparin. This finding is in agreement with previous studies (39) which...
have shown that heparin blocks the biological effect of basic FGF. Neither the number nor the molecular weight of these labeled species changed when the gels were run under reducing conditions. This indicates that they are not a part of a higher molecular weight complex composed of 2 subunits linked by disulfide bridges. It is possible that the lower molecular weight species may correspond to a degradation product of the $M_r = 145,000$ receptor species that would pre-exist in the intact cells or occur during incubation with $^{125}$I-FGF. That both receptor species are closely related as far as FGF binding to them is concerned is supported by the following observations. First, when the cross-linking is performed with decreasing concentrations of $^{125}$I-FGF (as low as a third of the dissociation constant value), the cross-linking of $^{125}$I-FGF to both proteins is decreased to approximately the same extent. This indicates that both polypeptides carry binding sites which have approximately the same affinity towards $^{125}$I-FGF. The cross-linking of $^{125}$I-FGF to both polypeptides is also decreased to the same extent when increasing concentrations of FGF are employed to chase the labeled probe from the cell surface binding site. This indicates that both macromolecular species have the same affinity for native FGF. Second, in some cross-linking experiments, the intensity of the lower labeled band seemed lower than that of the upper one (for example, see Fig. 10, lanes C to F). This could be the result of different rates of proteolysis in different experiments.

The structural relationship between the two $^{125}$I-FGF cross-linked macromolecular species is presently unknown. Use of monoclonal antibodies directed against the FGF receptor or peptide mapping should be useful in resolving this issue.

The molecular weight of the two macromolecular species of the FGF receptors is distinct from that of the PDGF receptor ($M_r = 200,000$ or $160,000$ and 110,000, Refs. 35, 40, and 41) $\beta$TFG receptor ($M_r = 280,000$, Ref. 29), $\alpha$TGF receptor ($M_r = 170,000$ and 60,000, Refs. 35 and 34), EGF receptor ($M_r = 170,000$ to 180,000, Ref. 42), and insulin receptor ($M_r = 350,000$ and 290,000, Ref. 43).

Tyrosine phosphorylation of the FGF receptor could not be detected even when reagents as sensitive as antibodies directed against phosphorylated tyrosine were used.$^3$ Threonine and serine phosphorylation of the FGF receptor were not examined in the present study. These phosphorylation sites could be more relevant to the biological activity of FGF than tyrosine phosphorylation, since this mitogen has been shown to activate protein C kinase (13). This raises the possibility that the FGF receptor, by analogy with the EGF receptor (44, 45), could be a substrate for the Ca$^{2+}$/phospholipid-dependent, threonine-specific protein C kinase. Within that context, it should be noted that others have reported that in A-431 cells, protein C kinase mediated threonine and serine phosphorylation of the EGF receptor leads to a diminished ability of EGF to stimulate its tyrosine protein kinase activity (44, 46). If it is also true for FGF, it could explain our inability to observe tyrosine phosphorylation of the FGF receptor.

In conclusion, the studies reported here suggest that the physiologically relevant FGF receptor consists of two macromolecular binding components. Preliminary studies done with vascular capillary endothelial cells show the existence of similar FGF receptors.$^4$ Obviously, other FGF receptor components could exist that interact with this binding species and become dissociated upon solubilization and electrophoresis of affinity labeled cells. Further characterization and isolation of the FGF receptors is needed to address this and other questions on their structure. The information derived from the present studies should help initiate efforts to quantitatively solubilize and isolate this receptor type.

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