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Visualization of Several Binding Sites for Basic Fibroblast Growth Factor (FGF-2) on Fibroblasts by Photoaffinity Labeling: Evidence for Intracellular Complexes

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Abstract The internalization of basic fibroblast growth factor (FGF-2) was studied in Chinese hamster lung fibroblasts (CCL39). Recombinant FGF-2 was derivatized with a photoactivable agent, N-hydroxysuccinimidyl-4-azidobenzoate (HSAB), iodinated, and used to visualize intracellular FGF-2-affinity-labeled molecules after internalization at 37°C. Iodinated HSAB-FGF-2 maintained the properties of natural FGF-2 such as affinity for heparin, binding to Bek and Flg receptors, interaction with high- and low-affinity binding sites, and reinitiating of DNA synthesis in CCL39 cells. Affinity-labeling experiments at 4°C with ^{125}I-HSAB-FGF-2 led to the detection of several FGF–cell surface complexes with apparent molecular mass of 80, 100, 125, 150, 170–180, 220, 260, and about 320 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), whereas two specific bands at 80 and 130–160 kDa were obtained using the homobifunctional cross-linking reagent, disuccinimidyl suberate. When the cells, preincubated with ^{125}I-HSAB-FGF-2 at 4°C and then washed, were shifted to 37°C, irradiation of the internalized labeled FGF-2 led to detection of a similar but fainted profile with one major specific band at 80 kDa. Heparitinase II treatment of the cells reduced binding of ^{125}I-HSAB-FGF-2 to its cell surface sites by 80% and internalization by 55%, indicating the involvement of heparan sulfate proteoglycans in these processes. Among the heparitinase-sensitive bands was the 80-kDa complex.

Key words: FGF, receptors, internalization, photoactivable cross-linker, heparan sulfate proteoglycans

Basic fibroblast growth factor (FGF-2) belongs to the family of nine heparin-binding proteins [reviewed in Basilico and Moscatelli, 1992; Baird, 1994]. FGF-2 is a potent mitogen for various cell types and stimulates cell migration and differentiation of mesenchymal and neuroectodermal cells in vitro [reviewed in Gospodarowicz et al., 1987; Rifkin and Moscatelli, 1989]. Radioreceptor-binding studies using iodinated FGF-2 on various cell types (fibroblasts, myoblasts, epithelial, endothelial, and neuronal cells), and affinity-labeling experiments have led to the characterization of cell surface receptors [Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986; Moenner et al., 1986; Moscatelli, 1987; Walicke et al., 1989]. Several glycoproteins that fulfill the characteristics of FGF receptors have been cloned on the basis of their tyrosine kinase domains [reviewed in Partanen, 1992]. Up to now, sequencing data have shown the existence of four families, each containing numerous variants. A non-tyrosine kinase FGF receptor has also been purified and cloned [Burrus et al., 1992].

Lower-affinity binding sites for FGF-2 have been identified on the cell surface and extracellular matrix (ECM) [Moenner et al., 1987; Moscatelli, 1987; Vlodavsky et al., 1987; Bashkin et al., 1989; Saksela and Rifkin, 1990]. These sites

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represent a family of heparan sulfate proteoglycans (HSPGs) exemplified by syndecan [reviewed in Bernfield et al., 1992; Chernousov and Carey, 1993] and perlecan [Aviezer et al., 1994]. Indirect roles have been proposed for HSPGs in FGF activity, such as stabilization and storage of the growth factor [Gospodarowicz and Cheng, 1986], protection against protease degradation [Saksela et al., 1988] and tissue diffusion [Flaumenhaft et al., 1990; Brunner et al., 1991]. It has been reported that HSPGs are essential for the binding of FGF-2 to high-affinity receptors [Rapraeger et al., 1991; Yayon et al., 1991; Aviezer et al., 1994], although other studies suggest that it may not be the case [Kiefer et al., 1991; Roghani et al., 1994]. However, it seems clear that HSPGs are involved in the biological response induces by the binding of FGF-2 to its high affinity receptors [Rapraeger et al., 1991; Yayon et al., 1991; Aviezer et al., 1994].

In a previous study, we have shown that FGF-2 internalization by CCL39 fibroblasts progressed through different pathways, depending on the FGF-2 concentration and involved high- and low-affinity binding sites [Gannoun-Zaki et al., 1991a]. Heparan sulfates were shown to take part in the internalization process [Roghani and Moscatelli, 1992; Reiland and Rapraeger, 1993; Rusnati et al., 1993; Quarto and Amalric, 1994]. To better understand the mechanism of FGF-2 interaction with its high- and low-affinity receptors, as well as define the cellular components implicated in internalization, we prepared a photoactivable derivative of FGF-2 and studied its binding and internalization in CCL39 fibroblasts by photoaffinity labeling. Photoactivable cross-linkers offer two advantages compared to chemical bifunctional reagents. First, the labeling of neighboring sites, including hydrophobic sites, should be possible, since the highly reactive nitrenes produced upon photolysis can react with CH groups [Ruoho et al., 1984]. Second, cross-linking of FGF-2 to associated molecule should be feasible at any time during FGF-2 internalization and give an image of interacting molecules in the intracellular pathways.

We report here the presence of several specific binding sites at the cellular surface or located inside the cell which were covalently linked to FGF-2 by photoaffinity labeling. The binding sites were heparitinase sensitive, suggesting that HSPGs were involved in FGF-2 internalization.

MATERIALS AND METHODS

Materials

Recombinant FGF-2 (FGF-2) was a gift from G. Mazue (Carlo Erba, Farmitalia). HSAB (N-hydroxysuccinimidyl-4-azidobenzoate) and DSS (disuccinimidyl suberate) were obtained from Pierce Chemical Co. (Rockford, IL). Heparitinase II, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (Hepes), bovine serum albumin (BSA) and protein A–Sepharose were purchased from Sigma (St. Louis, MO). Chemicals for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and molecular-weight markers were obtained from BioRad (Hercules, CA) and acrylamide from Serva (Heidelberg, Germany). Culture chemicals were purchased from Gibco BRL (Gaithersburg, MD) and fetal bovine serum (FBS) from Eurobio (Toulouse, France). Carrier-free ^{125}I-NaI and [methyl-^{3}H]thymidine were obtained from Oris (Gif-Sur-Yvette, France).

Cell Culture

Chinese hamster lung fibroblasts (CCL39) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown from passage 6–20 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 26 mM sodium bicarbonate, in 5% CO_2–95% air at 37°C, plated at 20,000 cells/cm^2 in 6-well dishes (Costar, Brumath, France) for the FGF-2 binding experiments. National Institutes of Health (NIH) 3T3 cells transfected with expression vectors containing the full coding sequence of FGF receptors; Bek (NBek8) and Flg (NFlg26) were a gift from M. Jaye [Dionne et al., 1990]. NBek8 and NFlg26 cells were grown in DMEM supplemented with 10% FBS, 26 mM sodium bicarbonate, and 500 μg/ml geneticin. Cells were plated at 40,000 cells/cm^2 in 6-well dishes.

Preparation of ^{125}I-HSAB-FGF-2

The photoactivable reagent (HSAB) was protected from light throughout the procedure. A fresh stock solution of HSAB (175 mM) in dimethyl sulfoxide (DMSO) was diluted in phosphate-buffered saline (PBS): 140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) and added at a 5g μM final concentration to 1.5 μM FGF-2, 1% polyethylene glycol

(PEG) 1,000 and 0.1 M phosphate buffer pH 7.4 in a polypropylene tube (100 μl final volume). After 30-min incubation at room temperature, 0.6 mCi ^{125}I-NaI and a 10-μl solution of chloramine T (800 μM in 0.5 M phosphate buffer, pH 7.4) were added. Samples were then mixed by pipetting for 2 min at room temperature. The reaction was stopped by adding 12.5 mM dithiothreitol for 3 min. Affinity chromatography was performed on a 200-μl heparin–Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with PBS containing 0.65 M NaCl and 1 mg/ml BSA. Derivatized FGF-2 was then eluted with 2 ml of PBS, 2.15 M NaCl, 1 mg/ml BSA, pH 7.4. ^{125}I-FGF-2 was prepared as previously described [Gannoun-Zaki et al., 1991a]. Specific radioactivity of ^{125}I-HSAB-FGF-2 and ^{125}I-FGF-2 was 100,000–300,000 cpm/ng. Biological activity was assessed by measuring DNA synthesis reinitiation in quiescent CCL39 cells.

Measurement of DNA Synthesis Reinitiation

Subconfluent CCL39 cells in 48-well plates were arrested in G0/G1 by a 24-h incubation in a serum-free medium. ^{125}I-HSAB-FGF-2, ^{125}I-FGF-2, or FGF-2 was then added for 24 h and [^{3}H]thymidine (0.5 μCi/ml) for the last 4 h. [^{3}H]Thymidine incorporation was determined as the remaining radioactivity associated with the cells after an incubation of 20 min at 4°C in a 10% solution of trichloroacetic acid and five washes with water. Radioactivity was quantified by liquid scintillation counting of the cells solubilized in 0.3 N NaOH. When carried out with ^{125}I-HSAB-FGF-2, this assay was performed in the dark, in order to protect the cross-linker from ultraviolet (UV) activation.

Radioreceptor Experiments

Binding constants for ^{125}I-HSAB-FGF-2 and ^{125}I-FGF-2 were determined at 4°C by saturation experiments on CCL39 cells and displacement experiments on bovine brain membrane preparations. Subconfluent CCL 39 cells (80,000–150,000 cells/cm^2) were cooled to 4°C for 30 min before being washed 3 times with 1 ml Hepes-buffered saline (HBS: 20 mM Hepes, 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing 1 mg/ml BSA. Increasing concentrations of ^{125}I-HSAB-FGF-2 or ^{125}I-FGF-2 and FGF-2 were added, and the cells were incubated for 3 h at 4°C under gentle agitation. Cells were washed 3 times in HBS containing 1 mg/ml BSA, solubilized in HBS containing 1% Triton X-100, and counted using a γ counter (LKB, Wallac, Turku, Finland).

On bovine brain membrane preparations [Ledoux et al., 1989], 50 μg of protein was incubated for 60 min at 4°C with 200 pg (23 pM) of ^{125}I-HSAB-FGF-2 or ^{125}I-FGF-2 in the presence of increasing concentrations of unlabeled FGF-2, to a final volume of 500 μl of a buffer containing 20 mM Hepes, 0.3 M sucrose, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1 M NaCl, 0.5% BSA, and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 5 kallikrein inhibitor U/ml (KIU/ml) aprotinin, 0.1 mM PMSF), pH 7.4. After incubation, bound and free FGF-2 were separated by centrifugation (1,100*g*, 5 min at 4°C). Radioactivity of the resulting pellet containing bound FGF-2 was counted in a γ counter. Analyses of the binding data were performed using the LIGAND fitting program [Munson and Rodbard, 1980].

Affinity Labeling of CCL39 Cells at 4°C, Using ^{125}I-FGF-2 and DSS

CCL39 cells were grown to subconfluency in 35-mm dishes. After washing three times in a HBS containing 1 mg/ml BSA and preincubating at 4°C for 20 min, ^{125}I-FGF-2 was added for 3 h at 4°C in the presence or absence of unlabeled FGF-2. The cells were then washed three times in HBS and incubated for 15 min in the same medium containing 0.25 mM DSS. The reaction was stopped by adding 10 mM methylamine. Cells were scraped in 1 ml of HBS containing 1 mg/ml BSA and protease inhibitors as above. After 1-min centrifugation at 1,100*g*, the pellets were solubilized in a Laemmli sample buffer (80 mM Tris–HCl, 2% SDS, 10% glycerol, 0.004% Bromophenol blue, 5% β-mercaptoethanol, pH 6.8), heated 5 min at 90°C and analyzed by gradient polyacrylamide SDS–PAGE and autoradiography.

Affinity Labeling of CCL39 Cells at 4°C, Using ^{125}I-HSAB-FGF-2

Subconfluent cultures of CCL39 fibroblasts in 35-mm dishes were washed three times in HBS, 1 mg/ml BSA and preincubated for 20 min at 4°C. ^{125}I-HSAB-FGF-2 was then added at different concentrations, and incubation was carried out for 3 h at 4°C. The specificity of ^{125}I-HSAB-FGF-2 binding was estimated by adding a 100-fold excess of unlabeled FGF-2. Cells were then

washed five times in HBS and irradiated at 254 nm for 7 min, 10 cm from the lamp (DESAGA, 2 × 8 w). The cells were then treated as above.

Immunoprecipitation of Bek and Flg Receptors

For immunoprecipitation studies using antireceptor antibodies, cross-linking experiments were carried out at 4°C with 10 ng/ml of ^{125}I-HSAB-FGF-2 on CCL39, NBek8, and NFlg26 cells. The cells were then scraped and centrifuged at 1,100*g* for 5 min. The pellets were incubated in 500 µl of HNTG buffer (20 mM Hepes, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 µg/ml of each aprotinin and leupeptin, 1 mM PMSF, pH 7.5) for 30 min at 4°C and centrifuged 5 min at 1,100*g*. Three mg of protein A–Sepharose was swollen and washed in 0.2 M borate buffer, pH 8.2, then mixed with anti-Bek 1 or anti-Flg 1 for 30 min at room temperature. The rabbit antipeptide antibodies were a gift from M. Jaye [Dionne et al., 1990]. The gel was washed three times in a HNTG buffer containing 0.1% Triton X-100 and incubated with the clarified cell lysates for 2 h at 4°C. It was then washed three times with a HNTG buffer containing 0.1% Triton X-100 and once in 10 mM Tris–HCl, pH 6.8. A Laemmli sample buffer was added to the gel and the mixture heated for 5 min at 90°C. Finally it was centrifuged at 1,100*g* for 1 min and the supernatant subjected to SDS–PAGE and autoradiography.

Photoaffinity Labeling of CCL39 Cells After Internalization of ^{125}I-HSAB-FGF-2 at 37°C

Internalization experiments were performed in CCL39 cells, with ^{125}I-HSAB-FGF-2, as described in the figure legends. Cell cultures were first incubated in the presence of ^{125}I-HSAB-FGF-2 at 4°C, washed and then shifted to 37°C. The cells were washed with 1 ml of acidic buffer (0.2 M acetic acid, 0.5 M NaCl, pH 2.5) [Haigler et al., 1980] for 5 min, to discriminate between intra- and extracellular FGF-2 [Gannoun-Zaki et al., 1991a] and then irradiated as described above. This acid wash was shown to dissociate at least 95% of FGF-2 bound to the cell-membranes. The cells were scraped with a rubber policeman in a HBS buffer containing 1 mg/ml BSA, 1 µg/ml of each leupeptin and pepstatin, 1 mM PMSF and 5 KIU/ml of aprotinin, collected by centrifugation at 1,100*g* for 5 min, solubilized in a Laemmli sample buffer and analyzed by SDS–PAGE as described.

Heparitinase II Treated Cells

Subconfluent CCL39 cells (125,000 cells/cm^2) were pretreated for 1 h at 37°C with 0.5 U/ml heparitinase II in 20 mM Hepes, 130 mM NaCl, 0.68 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.1 mg/ml BSA, 0.1 mM PMSF, 1 µg/ml leupeptin, and 5 KIU/ml aprotinin, pH 7.0. The cells were then rinsed three times in HBS, 1 mg/ml BSA and incubated with 7 ng/ml of ^{125}I-HSAB–FGF-2 for 3 h at 4°C. They were washed 5 times in HBS buffer before being subjected to UV irradiation or shifted to 37°C for 30-min incubation. The cells incubated at 4°C were washed in HBS, 1 mg/ml BSA and solubilized with a sample buffer. The cells incubated at 37°C were first rinsed with an acidic buffer before solubilization. Radioactivity bound to the cells at 4°C or internalized at 37°C was quantified. The samples were finally submitted to SDS–PAGE.

SDS–PAGE and Autoradiography

Gel electrophoresis was performed according to Laemmli [1970], using a 3% polyacrylamide stacking gel and a 3–10% or 5–10% gradient polyacrylamide running gel. After electrophoresis, the gels were stained with R250 Coomassie blue (0.25% w/v, acetic acid–methanol–water, 14:50:36) and destained in a solution of acetic acid–methanol–glycerol–water (10:30:5:55). Autoradiograms (Kodak X-Omat R films) were obtained from the dried gel after exposure for the indicated period of time. The molecular-mass markers were myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (92.4 kDa), BSA (66.2 kDa), and ovalbumin (45 kDa).

RESULTS

Characteristics of ^{125}I-HSAB-FGF-2

Iodinated HSAB-FGF-2 was retained on a heparin–Sepharose column equilibrated in PBS, 0.65 M NaCl to the same extent (70–85%) as ^{125}I-FGF-2 and unlabeled FGF-2, indicating that the affinity of the growth factor for heparin was not significantly modified by coupling the photoreactive group and iodine to FGF-2.

[^{3}H]Thymidine incorporation in CCL39 stimulated cells by ^{125}I-HSAB-FGF-2 was obtained with an effective concentration necessary for 50% activation (EC_{50}) at 0.3 ng/ml, as compared to 0.45 ng/ml for FGF-2 (Fig. 1) and 0.5 ng/ml

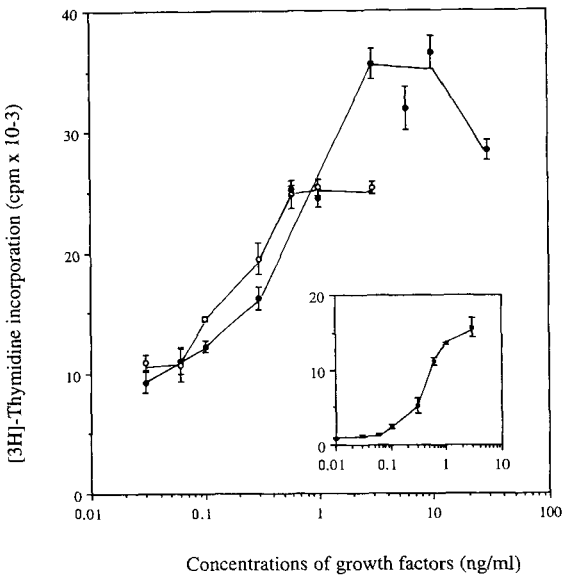

Fig. 1. Dose-response curves showing DNA synthesis reinitiation induced by ^{125}I-HSAB-FGF-2, FGF-2, and ^{125}I-FGF-2 in CCL39 cells. Cells were arrested in G0 phase by a 24-h incubation in a serum-free medium, then the growth factors were added for 24 h and [^{3}H]thymidine (0.5 μCi/ml) for the last 4 h. Thymidine incorporation was quantified as described in Methods. ○, ^{125}I-HSAB-FGF-2; ○, FGF-2; inset, ●, ^{125}I-FGF-2.

for ^{125}I-FGF-2 (Fig. 1 inset). The maximal stimulatory effect was observed with 1 ng/ml of ^{125}I-HSAB-FGF-2 and 3 ng/ml of FGF-2 or ^{125}I-FGF-2.

The binding capacity of ^{125}I-HSAB-FGF-2 to FGF-2 receptors and low affinity sites was assessed on CCL39 cells (Table I). Routinely, the iodinated derivative was tested on bovine brain membrane preparations that contained high- and low-affinity binding sites for FGF-2 [Ledoux et al., 1989]. Ligand analysis of the binding data using ^{125}I-HSAB-FGF-2 and ^{125}I-FGF-2, respectively, resulted in curvilinear Scatchard plots suggesting the existence of at least two families of interactions on cells and membrane preparations, respectively. The apparent dissociation constants ($appK_d$) and binding capacity presented in Table I were within the same range for both ^{125}I-HSAB-FGF-2 and ^{125}I-FGF-2 (Student's t-test with $P < 0.0001$).

Thus, ^{125}I-HSAB-FGF-2 and ^{125}I-FGF-2 showed similar capacities to induce biological activity in the target cells and to bind to membrane receptors. Therefore, the photoactivable derivative could be used for affinity labeling of binding components at the cell surface and at the intracellular level during internalization.

Affinity Labeling Experiments on CCL39 Cells

Affinity labeling experiments on CCL39 cells were carried out at 4°C using either ^{125}I-HSAB-FGF-2 or ^{125}I-FGF-2 in the presence of DSS.

As presented in Figure 2A, several complexes migrating in SDS–PAGE at 80, 100, 125, 150, 170–180, 220, 260, and about 320 kDa were obtained by photoactivation of the heterobifunctional reagent (HSAB) coupled to FGF-2. Most of these bands were not detectable in the presence of a 100-fold excess of FGF-2. Only the 80-, 125-, and 320-kDa bands, which originally were the most intense, could be detected, but as a considerably reduced signal.

Affinity-labeling experiments using 1 ng/ml ^{125}I-FGF-2 and the homobifunctional chemical cross-linker (DSS), resulted in the formation of fewer specific complexes with a molecular mass of 130–160 kDa (Fig. 2B) as described for other cell lines [Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986, Moenner et al., 1987] and 80 kDa. These complexes have been obtained previously using 5 ng/ml ^{125}I-FGF-2 on CCL39 cells [Gannoun-Zaki et al., 1994]. Thus, photoactivable derivatized FGF-2 labeled several specific high- and low-affinity binding entities that were not visualized using the chemical reagent, DSS.

Immunoprecipitation of Photoaffinity-Labeled Cell Surface Receptors

Northern blot analysis of CCL39 mRNA using Bek and Flg probes indicated that these cells weakly expressed both receptor transcripts [Gannoun-Zaki et al., 1994]. Thus, the question was raised about the presence of these receptors among the different complexes obtained using ^{125}I-HSAB-FGF-2.

Immunoprecipitation experiments were carried out using anti-Bek and anti-Flg antibodies. Transfected cells, NBek8 and NFlg26, expressing high numbers of Bek and Flg receptors (64,000 and 55,000, respectively [Dionne et al., 1990]), were subjected to affinity labeling experiments as controls. Bek and Flg anti-peptide antibodies immunoprecipitated complexes migrating in SDS–PAGE as 130- and 150-kDa-labeled bands from NBek8 and NFlg26 cells, respectively (Fig. 3A). These bands were not detected by preimmune serum (lanes 3). These data indicated that ^{125}I-HSAB-FGF-2 interacted with Bek and Flg receptors.

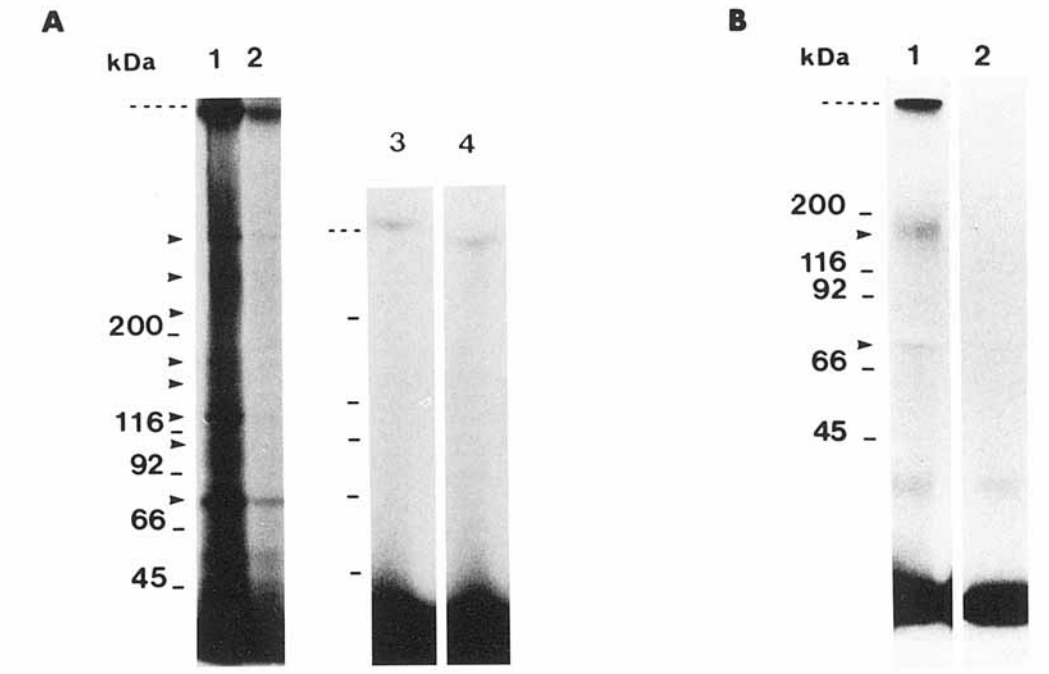

Fig. 2. Affinity-labeling experiments using ^{125}I-HSAB-FGF-2 (**A**) and ^{125}I-FGF-2 and DSS (**B**) on CCL39 cells at 4°C, in the absence (*lanes 1, 3*) or in the presence (*lanes 2, 4*) of a 100-fold (**A**) and 200-fold (**B**) excess of FGF-2. **A:** CCL39 cells (95,000 cells/cm^2) were incubated with 7 ng/ml of ^{125}I-HSAB-FGF-2. After 3-h incubation at 4°C, the cells were washed five times and subjected to UV irradiation at 254 nm for 7 min (*lanes 1, 2*). Controls without irradiation, *lanes 3, 4*. The cells were then scraped with a rubber policeman and analyzed by a 3–10% gradient PAGE. The bands were revealed after 15 days exposure. **B:** 1 ng/ml of ^{125}I-FGF-2 was bound to CCL39 cells (60,000 cells/cm^2) for 3 h at 4°C. After washing, 0.25 mM DSS was added for 15 min at 4°C, and the cross-linking reaction was stopped by the addition of 10 mM methylamine. The cells were scraped, solubilized in a Laemmli sample buffer and analyzed by a 5–10% gradient SDS–PAGE. The autoradiogram shown was obtained after a 2-day exposure of the dried gel. Bands corresponding to cross-linked complexes (*arrowheads*).

TABLE I. Binding Constants for ^{125}I-HSAB-FGF-2 and ^{125}I-FGF-2 on CCL39 Cells and Bovine Brain Membrane Preparations at 4°C Using the Curve-Fitting LIGAND Program

	High-affinity sites		Low-affinity sites	
	appK$_d$ (pM)	Binding capacity	appK$_d$ (nM)	Binding capacity
Cells[a]		Sites/cell		Sites/cell
^{125}I-HSAB-FGF-2	5 ± 2	500 ± 150	3 ± 2	5 10^5 ± 3 10^5
^{125}I-FGF-2	10 ± 5	3,000 ± 1,400	10 ± 5	10^6 ± 0.5 10^6
Brain membranes[b]		fmoles/mg protein		pmoles/mg protein
^{125}I-HSAB-FGF-2	276 ± 47	3.0 ± 0.6	13 ± 5	34 ± 9
^{125}I-FGF-2	435 ± 200	2.8 ± 1.7	11 ± 5	56 ± 18

[a]Subconfluent CCL39 cells (about 100,000 cells/cm^2) in 6-well plates were incubated with increasing concentrations of ^{125}I-HSAB-FGF-2 or ^{125}I-FGF-2 and FGF-2 for 3 h at 4°C. Bound radioactivity was determined after 3 washes as described under Materials and Methods.

[b]Membrane proteins (50 μg) were incubated for 60 min at 4°C with 200 pg of ^{125}I-HSAB-FGF-2 or ^{125}I-FGF-2 in the presence of increasing concentrations of unlabeled FGF-2. Bound radioactivity was determined as described under Materials and Methods.

The SDS–PAGE profile obtained with CCL39 cells was different from that obtained with Bek- and Flg-transfected cells, respectively (Fig. 3A, lanes T, N). Furthermore, immunoprecipitation of the complexes was not observed under the same conditions (Fig. 3A). An affinity-labeled molecule immunoprecipitated by anti-Bek antibodies, was detected at 130–150 kDa after 40-day exposure of the autoradiogram (Fig. 3B, lane 1). Thus, HSAB-derivatized FGF-2 could

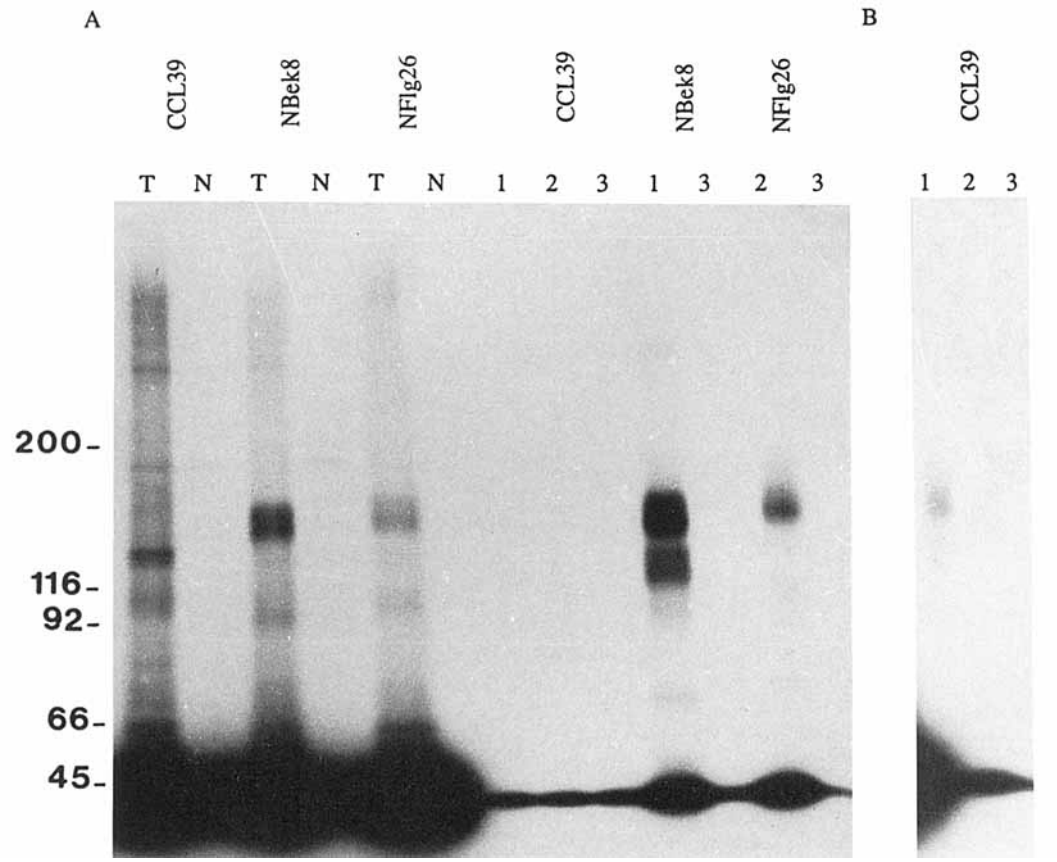

Fig. 3. Immunoprecipitation of ^{125}I-HSAB-FGF-2 linked to the cell surfaces using Bek and Flg anti-peptide antibodies. CCL39 cells (205,000 cells/cm^2), NBek8 cells (100,000 cells/cm^2), NFlg26 cells (75,000 cells/cm^2) were incubated with 10 ng/ml of ^{125}I-HSAB-FGF-2 for 2 h at 4°C. Cross-linking experiments were performed as described above in the absence (T) or in the presence (N) of a 100-fold excess of FGF-2. Samples were either subjected to SDS–PAGE (T, N) or immunoprecipitated by anti-Bek (*lane 1*), anti-Flg (*lane 2*) or nonimmune serum (*lane 3*) and then analyzed on SDS–PAGE. Autoradiographs presented were obtained after 7 days (**A**) or 40 days (**B**) of exposure.

be cross-linked to different binding molecules on CCL39 cells including Bek receptors.

Photoactivation of ^{125}I-HSAB-FGF-2 Internalized in CCL39 Cells at 37°C

In order to visualize intracellular components bound to FGF-2, internalization experiments were performed. Binding was performed at 4°C in the presence of 400 pM (7 ng/ml) ^{125}I-HSAB-FGF-2; the cells were then washed and shifted to 37°C for 30 min to induce internalization. Extracellular bound FGF-2 was washed out at pH 2.5; the cells were then irradiated. SDS–PAGE autoradiography of the irradiated cells (Fig. 4A) showed a similar and less intense profile than that obtained by incubating the cells at 4°C (Figs. 2A, 4A) with one major specific complex migrating at 80 kDa. The similarity of the profiles obtained at 37°C and 4°C suggested that FGF-2 and these extracellular binding sites were internalized. The 80-kDa complex that represented 40% of the affinity-labeled bands at 4°C (Fig. 2A), accounted for 90% of the internalized radioactivity at 37°C (Fig. 4A).

Binding and Internalization of ^{125}I-HSAB-FGF-2 in Heparitinase II-Treated CCL39 Cells

FGF-2 has been reported to bind HSPGs (Moscatelli, 1987; Vigny et al., 1988; Saksela and Rifkin, 1990). In order to assess the involvement of HSPGs in the binding and internalization of FGF-2, CCL39 cells were pretreated with heparitinase II for 1 h at 37°C, and binding and internalization experiments were then carried out. Figure 4B shows that 80% of the total bound ^{125}I-HSAB-FGF-2 at 4°C was prevented in cells pretreated with heparitinase II. Internalization of FGF-2 also appeared to be sensitive to heparitinase II pretreatment, since it was reduced by 55%. These results suggested that

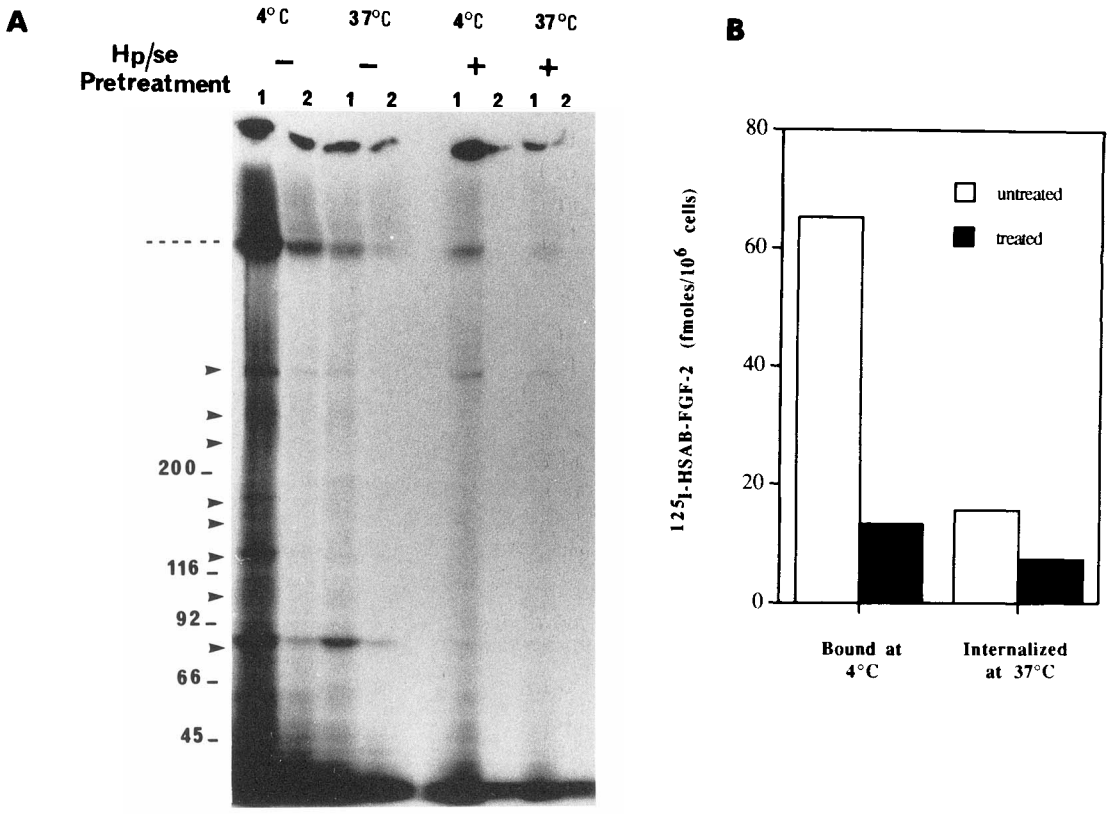

Fig. 4. Cross-linking and binding (**A, B**) of ^{125}I-HSAB-FGF-2 to untreated or heparitinase II-treated CCL39 cells. **A:** Cross-linking experiments of ^{125}I-HSAB-FGF-2 to CCL39 cells untreated (−) or pretreated (+) with heparitinase II. The cells were incubated at 4°C with 7 ng/ml ^{125}I-HSAB-FGF-2 in the absence (*lanes 1*) or in the presence (*lanes 2*) of a 100-fold excess of FGF-2. CCL39 cells (125,000 cells/cm^2) were either irradiated at 254 nm for 7 min (4°C) or shifted to 37°C for 30 min before being washed with an acidic buffer and irradiated (37°C). They were analyzed by SDS–PAGE. *Arrowheads,* bands corresponding to various cross-links. . . . , origin of the running gel. **B:** Cells were untreated (open bar) or pretreated (solid bar) with 0.5 U/ml of heparitinase II for 1 h at 37°C. After cell washing, 7 ng/ml ^{125}I-HSAB-FGF-2 was added for 3 h at 4°C (4°C), or 3 h at 4°C, followed by 30 min at 37°C. Cells incubated at 4°C were scraped and solubilized with a sample buffer. The cells incubated at 37°C were first rinsed with an acidic buffer before solubilization. Radioactivity cell associated at 4°C or internalized at 37°C was quantified, respectively. Values are the means of duplicate determinations that varied at less than 10%.

heparan sulfate structures involved in FGF-2 binding were also partially involved in the internalization process. Among the specific complexes obtained by cross-linking ^{125}I-HSAB-FGF-2 to CCL39 cells at 4°C and still observed after the heparitinase pretreatment, only the high-molecular-weight complex at about 320 kDa was clearly present, whereas traces of the 80-kDa band were also detected (Fig. 4A). After internalization at 37°C, these bands were still faintly detected. These data showed that the formation of the 80-kDa complex was heparitinase sensitive and suggested that this structure contained heparan sulfate chains.

DISCUSSION

Previous studies of the FGF-2 internalization in Chinese hamster lung fibroblasts (CCL39 cells) have indicated that the mechanism was heterogeneous and involved different pathways, depending on the FGF-2 concentration [Gannoun-Zaki et al., 1991a]. To better understand the involvement of high- and low-affinity FGF-2 binding sites in the internalization process, FGF-2 was coupled to a photoactivable reagent, N-hydroxysuccinimidyl-4-azidobenzoate (HSAB). After iodination, the derivatized FGF-2 (HSAB-FGF-2) displayed the same properties as the native FGF-2 in inducing DNA reinitiation in CCL39 cells and binding to high-affinity receptors and low-affinity binding sites.

Compared to affinity-labeling experiments using ^{125}I-FGF-2 and the chemical reagent DSS, photoactivation of bound ^{125}I-HSAB-FGF-2 to CCL39 cells resulted in the formation of more complexes with apparent molecular mass of 80,

100, 125, 150, 170–180, 220, 260, and about 320 kDa, suggesting the involvement of different CCL39 cell surface entities that were part of or close to FGF-2 binding sites. This finding also suggests that free amines may not be available to react with the homobifunctional cross-linker DSS. In addition, the distance between the two reactive groups in HSAB is 8 Å, as compared to 11 Å in DSS, and therefore cross-links with HSAB represent closer interactions. Immunoprecipitation experiments with anti-Bek receptor antibodies revealed very weak cross-linked complexes on CCL39 cells of 130–150 kDa, as compared to those obtained using Bek-transfected cells, NBek8 cells. The specificity of these interactions was assessed by competition experiments with a 100-fold excess of unlabeled FGF-2. High- and low-affinity sites on CCL39 cells have appK_d of 10 pM and 10 nM, respectively [Gannoun-Zaki et al., 1991a], the ^{125}I-HSAB-FGF-2 concentration used (7 ng/ml = 400 pM) led to the saturation of high-affinity receptors and to partial occupancy of the low-affinity sites. Furthermore, the addition of a 100-fold excess of FGF-2 (40 nM) could fully displace the binding of ^{125}I-HSAB-FGF-2. Consequently, the complexes observed were likely to represent mainly low-affinity binding sites.

Because of the properties of the photoactivable reagent HSAB coupled to FGF-2, potential intracellular targets of the growth factor were investigated. Indeed, photoactivation of internalized ^{125}I-HSAB-FGF-2 in CCL39 cells resulted in the visualization of intracellular complexes whose molecular mass corresponded to those that were affinity-labeled on the cell surface at 4°C. However 90% of the internalized and cross-linked radioactivity was associated with the 80-kDa complex, which represented only 40% of the bands visualized after binding at 4°C (Fig. 4A). This could indicate that the 80-kDa band was more intimately localized in membrane when internalization was occurring.

It has been reported that in addition to high-affinity receptors, FGF-2 binds to HSPGs of ECM or cell surface [Jeanny et al., 1987; Moscatelli, 1987; Vlodavsky et al., 1987; Moscatelli, 1988; Bashkin et al., 1989]. Furthermore, HSPGs have been shown to be involved in the presentation of FGF-2 to high-affinity receptors [Yayon et al., 1991] in the mitogenic action of FGF-2 on fibroblasts and in FGF-induced differentiation of MM14 skeletal muscle cells [Rapraeger et al., 1991]. On CCL39 cells, 80% of FGF-2 binding sites were sensitive to heparitinase II treatment, and the major bands visualized by affinity labeling were abolished except for the 320-kDa band. This suggested that the photoactivable FGF derivative allowed the visualization of heparan sulfate-binding sites or alternatively sites whose recognition by FGF depended on heparan sulfate in their vicinity.

Since HSPGs, located at the cell surface or in the ECM, have been shown to be internalized by various cells [Bienkowski and Conrad, 1984; Iozzo, 1987], we could expect a direct role of HSPGs in FGF-2 internalization. Indeed, HSPGs associated or not to FGF-2 receptors have been shown to target FGF to different intracellular destinations [Reiland and Rapraeger, 1993]. They may even act as direct transducers of FGF signaling [Quarto and Amalric, 1994]. The internalization of ^{125}I-HSAB-FGF-2 decreased by 55% when CCL39 cells were pretreated with heparitinase II. These results indicated that FGF-2 internalization might also occur via HSPGs, in agreement with other studies [Gannoun-Zaki et al., 1991a; 1991b; Roghani and Moscatelli, 1992; Rusnati et al., 1993; Reiland and Rapraeger, 1993; Quarto and Amalric, 1994]. The major band of 80 kDa resulting from receptor internalization appeared to be heparitinase sensitive. Cross-linking experiments in the presence of increasing concentrations of unlabeled FGF-2 showed that the 80-kDa complex was the last to be displaced (not shown). These two points indicated that the 80-kDa complex resulted from a low-affinity/high-capacity interaction involving heparan sulfate molecules.

Disregarding the 130- to 160-kDa high-affinity receptor complex, poorly detected by photoaffinity labeling, the data obtained suggested that FGF-2 internalization involved a major heparan sulfate associated molecule that was also revealed by the chemical cross-linker DSS as a 80-kDa band. Further characterization of this molecule and its potential role in the biological function of FGF-2 remain to be determined.

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