Biochemical Pharmacology, Vol. 57, pp. 1399–1406, 1999.
© 1999 Elsevier Science Inc. All rights reserved.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00051-9

Effects of the Binding of a Dextran Derivative on Fibroblast Growth Factor 2: Secondary Structure and Receptor-Binding Studies

Patrick Bittoun, Rozita Bagheri-Yarmand,† Frédéric Chaubet,‡ Michel Crépin,† Jacqueline Jozefonvicz‡ and Serge Fermandjian*§*

*Département de Biologie Structurale, CNRS-UMR 1772, Villejuif; †Laboratoire de Recherche en Oncologie Moléculaire Humaine, Université Paris-Nord, Bobigny; and ‡Laboratoire de Recherches sur les Macromolécules, CNRS-UMR 7540, Université Paris-Nord, Villetaneuse, France

ABSTRACT. CMDB (carboxymethyldextran-benzylamide) are dextrans statistically substituted with carboxymethyl and benzylamide groups which can mimick some of the biological properties of heparin. It has previously been shown that CMDB inhibit autocrine growth of breast tumor cells (Bagheri-Yarmand *et al.*, *Biochem Biophys Res Commun* **239:** 424–428, 1997) and selectively displace fibroblast growth factor 2 (FGF-2) from its receptor. Here, we used circular dichroism and fluorescence anisotropy measurements to show that the conformation of FGF-2 was significantly altered upon its binding to CMDB and to short CMDB fragments prepared within this study. CMDB and fragments formed a stable 1:1 complex with FGF-2, with affinities being estimated as 20 ± 10 nM from fluorescence anisotropy analysis. No such a complex was formed with insulin-like growth factor (IGF-1) or epidermal growth factor (EGF). CMDB competed with the FGF-2 receptor for binding to FGF-2 but did not disturb the binding of IGF-1 and EGF to their receptors. Thus, our results highlight the selectivity of CMDB and their fragments towards FGF-2. Heparin, however, competes with CMDB and their fragments for binding to FGF-2. The carboxymethyl and benzylamide groups of these molecules likely interact directly with a heparin-binding region of FGF-2. The resulting change in conformation disturbs the binding of FGF-2 to its receptor and consecutively its mitogenic activity. BIOCHEM PHARMACOL **57**;12:1399–1406, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. CMDB; heparin-like; FGF-2; DTAF labelling; interactions; circular dichroism; fluorescence spectroscopy

FGF-2[‖] belongs to a large family of at least 13 growth factors that play an important role in the growth of cells from different origins. FGF-2, the best characterized member of this family of heparin-binding growth factors, participates in various physiological and pathological processes such as cellular differentiation and development [1], embryogenesis [2], and transformation and angiogenesis [3]. It strongly interacts at the cell surface with glycosaminoglycans, a class of FGF-2 low-affinity receptors [4, 5] required for the binding of growth factors to high-affinity receptors [4, 6]. The binding of FGF-2 to these low-affinity receptors induces a conformational change in the FGF-2 [4, 7] holding conformation that is more complementary to high-affinity receptors [8].

Heparin provides a storage and regulated release molecule for FGF-2 at the cell surface [9] as well as inducing FGF-2 conformational change, whereas other glycosaminoglycans such as chondroitin sulfate A, hyaluronic acid, or dextran are insufficient [10, 11]. FGF-2 selectivity towards heparin is influenced by the cluster of basic residues composing all heparin-binding proteins [12]. However, a basic domain is not sufficient in itself to promote glycosaminoglycan binding; non-ionic hydrogen interactions also seem to play a part [13, 14]. Integrity of the secondary and tertiary structures is also required [15].

Several authors have assessed the affinity of heparin and heparin derivatives for FGF-2 with various techniques, including fluorescence spectroscopy [11, 16–18]. Others have tried to define the minimal structural requirements of glycosaminoglycans that provide FGF-2 specific binding [17–21]. Data underline that O-sulfate groups at C2 and at carboxyl groups of iduronate residues are essential, and that a minimal length is required to obtain high-affinity binding to FGF-2 [19, 21, 22].

CMDB are statistically carboxymethylated and ben-

§ Corresponding author: Dr S. Fermandjian, Laboratoire de Physicochimie et Pharmacologie des Macromolécules Biologiques, Département de Biologie Structurale, CNRS-UMR 1772, Institut Gustave Roussy, 94805, Villejuif, France. Tel. (33-1) 42 11 49 85; FAX (33-1) 42 11 52 76; E-mail: sfermand@igr.fr

‖ *Abbreviations:* CMDB, carboxymethyldextran-benzylamide; FGF-2, basic fibroblast growth factor; FGF-1, acidic fibroblast growth factor; IGF-1, insulin-like growth factor; EGF, epidermal growth factor. DTAF, 5-([4,6-dichlorotriazine-2-yl]amino) fluorescein; CD, circular dichroism; and *f*-CMDB, fragmented CMDB.

Received 10 June 1998; accepted 14 December 1998.

zylaminated dextrans displaying some of the heparin properties [23–29]. These compounds inhibit human mammary tumor cell proliferation [24–26], while lacking the anticoagulant activity of heparin. Recent studies have shown that CMDB interfere with the FGF-2 autocrine loop in human breast epithelial HBL100 cells [1]. These results support the hypothesis that CMDB and their sulfated version CMDBS (carboxymethyldextran-benzylamide-sulfonate/sulfate) are candidates to be regulators of FGF-2 activity [30, 31]. For instance, previous work has shown that CMDB decrease the number of high- and low-affinity receptors in HBL100 cells without modulating FGF-2 receptor affinity [1].

Study of the structural and mechanistic aspects of CMDB binding to FGF-2 is needed to better understand the antiproliferative effect of these modified dextrans. We examined the differential capacity of the CMDB and of its fragments obtained in this work to bind FGF-2 or several other growth factors and to alter their conformations. The fragments still inhibit mammary tumor cell growth, with some being more potent inhibitors than others.*

The binding properties of CMDB, CMDB fragments, and heparin to FGF-2 and to other growth factors were assessed by both CD and fluorescence anisotropy, the latter requiring preparation of fluorescent CMDB. The selective binding of CMDB and their fragments to FGF-2 was discussed together with results obtained from FGF-2 receptor cross-linking experiments.

MATERIALS AND METHODS

Materials

Growth factors FGF-2, FGF-1, IGF-1, and EGF were purchased from Tebu. Standard heparin H410 (13,000 g/mol) was obtained from Institut Choay-Sanofi.

Cell Culture

Breast epithelial cell line HBL100 cells were routinely growth in Dulbecco's modified Eagle's medium (GIBCO) complemented with 10% fetal bovine serum (GIBCO), 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 IU-50 μg/mL penicillin–streptomycin mixture (GIBCO).

Synthesis

SYNTHESIS OF CMDB. CMDB were prepared from T40 dextran and characterized as previously described [27, 31, 32]. The final CMDB product was obtained with a degree of substitution in carboxymethyls (CM) and benzylamide (B) of 0.7 and 0.3, respectively.

PREPARATION OF DTAF-LABELED CMDB. In order to label CMDB with DTAF fluorochrome (Sigma), a small number of amino groups have been introduced after the carboxymethylation step, using bromopropylamine as previously described [33]. DTAF fluorochrome was added dropwise to the CMDB solution in borate buffer at pH9. After adjusting to pH7, the resulting labeled CMDB was eluted (G25 Sephadex column, Pharmacia), diafiltered under nitrogen pressure through cellulose acetate membrane (YM2, 1,000 g/mol, Amicon) and dialyzed against borate buffer on 6,000–8,000 D membrane, then recovered by lyophilization.

Fragmentation of CMDB and Labeled CMDB

Polymers were fragmented into low molecular weight products according to the method of Nardella *et al.* [34]. Fragmented CMDB, either labeled or not, are denoted *f*-CMDB. Molecular weights were determined by high-performance steric exclusion chromatography using a Lichrospher Si 300 diol column (Merck-Clevenot) and a Hema Sec Bio 40 column (Altech) connected in series to a 510 model pump (Merck) and to a Rheodyne injection valve with 100 μL loop. Columns were calibrated with standard polysaccharides, pullulans of various molecular weights (Polymer Laboratories, Interchim, Fluka), dextran, sucrose, and glucose (Sigma). Samples were eluted in an NaCl solution (0.15 M), Na_2HPO_4 (0.05 M, pH7.3) under refractometric monitoring (Jobin-Yvon, France). Peak molecular weight was determined using the GPC Chromstar software (Brüker, Merck-Clevenot). The chromatographic molecular weights were 80,000 g/mol for CMDB and 6,000 g/mol for the fragmented *f*-CMDB.

Fluorescence Experiments

Binding of labeled CMDB and *f*-CMDB to growth factors was monitored by alteration of the fluorescence properties of the DTAF probe bound to these molecules. Fluorescence spectra were recorded with a Jobin-Yvon Spex Fluororolog 1681 0.22 Spectrometrics with 2–10 nm resolution slit. Measurements of the fluorescence anisotropy at 517 nm were performed under physiological conditions and at 20° using an excitation wavelength of 488 nm (vertical scatter). Small volume aliquots of FGF-2 (8 μM) were added to a cuvette with a 1-cm path length containing 1 mL of the labeled CMDB sample (100 nM) in PBS. Incubation periods of 2 min were adopted to reach equilibrium. Data were corrected for dilution. Anisotropy was determined by $r = (V_V\text{-}GV_H)/(V_V + 2GV_H)$ where r, V, and H stand for anisotropy, vertical and horizontal detection under vertical excitation, respectively, and $G = V_V/V_H$ under horizontal excitation [35].

Circular Dichroism Experiments

CD spectra in the far-UV region were recorded on a CD6 Jobin-Yvon dichrograph. Measurements were performed at 20° in quartz cells of 0.1-mm path length for a 180 μL sample. Growth factor concentration varied from 1 to 5 μM

* Bittoun P, Chaubet F, Jozefonvicz J and Fermandjian S, unpublished results.

in a Tris buffer 10 mM NaCl, pH 7.4. Data were corrected for dilution. Titration of growth factors was performed by adding CMDB and heparin at adapted concentrations to obtain molar ratios of [compound]/[protein] ranging from 0 to 2 or from 0 to 10 for EGF.

Affinity Cross-linking Experiments

Subconfluent HBL100 cell cultures in 6-well tissue culture plates were washed twice with the binding buffer. Cells were incubated for 2.5 hr at 4° with ^{125}-I (iodinated) FGF-2, EGF, or IGF-1 (2.5 ng/mL) in the presence or absence of CMDB (50 μM), and competition experiments were performed in the presence of a 400- to 1,000-fold excess of unlabeled growth factor. Cells were then washed with ice-cold binding buffer without BSA and cross-linked for 20 min at room temperature with 0.25 mM disuccinimidyl suberate, prepared from a 25-mM stock solution in DMSO. The reaction was quenched by addition of an excess of 10 mM Tris-HCl pH 7.5/150 mM glycine for 5 min. Cells were then washed with ice-cold PBS, scraped in ice-cold PBS/aprotinin (1 μg/mL)/leupeptin (2 μg/mL)/0.1 mM phenylmethysulfonyl fluoride, centrifuged, and resuspended for 10 min at 4° in lysis buffer (50 mM Tris–HCl pH 8, 150 mM Nacl, 0.5 mg $MgCl_2$, 0.5% Nonidet P-40 v/v). Cell lysates were then clarified by centrifugation and supernatants mixed with sample buffer 2× (100 mM Tris–HCl, 4% SDS, 10% glycerol, 0.05% bromophenol blue) and boiled for 3 min before loading on an SDS-polyacrylamide gel (7.5 or 10%). After migration running, gels were stained with 0.25% Coomassie blue, destained with 10% acetic acid, 45% methanol, dried and processed for autoradiography by exposure (10–20 days) to a Kodak X-omat film at −80°.

RESULTS

We obtained a final CMDB product with a degree of substitution in carboxymethyl (CM) and benzylamide (B) of 0.7 and 0.3, respectively. CMDB (80,000 g/mol) and *f*-CMDB (6,000 g/mol) possessed the same overall composition for their CM and B groups. The DTAF-labeled CMDB were depolymerized to give the DTAF-labeled *f*-CMDB without any loss of fluorescence properties.

CMDB Inhibition of Cross-linked FGF-2 Receptors

As described in Materials and Methods, the FGF-2 receptor was characterized by cross-linking of radiolabeled FGF-2 to HBL100 cells. Our experimental conditions enabled us to detect a band of ≈150 kDa. In binding experiments, radiolabeled FGF-2 was used to specifically compete for radiolabeled FGF-2 (Fig. 1, lanes 1 and 2). The molecular weight of the receptor (130 kDa) determined after subtraction of the FGF-2 molecular weight was in agreement with that previously reported [36]. Incubation of HBL100 cells with CMDB (50 μM) led to a noticeable decrease in FGF-2

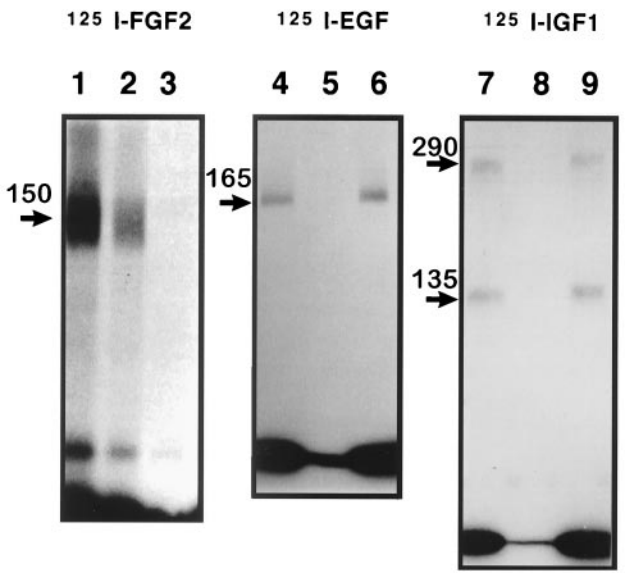

FIG. 1. Affinity cross-linking of ^{125}I-FGF-2, ^{125}I-EGF, and ^{125}I-IGF-1 to HBL100 cell surface. Subconfluent cultures of HBL100 cells were incubated with 2.5 ng/mL of ^{125}I-FGF-2 (lane 1), ^{125}I-EGF (lane 4), ^{125}I-IGF-1 (lane 7), and one of the following ligands, namely, FGF-2 (1,000 ng/mL, lane 2), EGF (500 ng/mL, lane 5), IGF-1 (500 ng/mL, lane 8), CMDB (50 μg/mL, lanes 3, 6, and 9). After cross-linking with disuccinimidyl suberate at 0.025 mM, cells were lysed and cell extracts subjected to electrophoresis on denaturating 7.5% polyacrylamide gels for EGF and IGF-1 and 10% polyacrylamide gels for FGF-2. The apparent molecular weights, in kDa, of the growth factor–receptor complexes are indicated by arrows.

receptor complex formation. The band intensities reflected an inhibition of FGF-2 binding to its specific receptor (Fig. 1, lane 3). At the same time, incubation of radiolabeled ^{125}I-IGF-1 and ^{125}I-EGF with the HBL100 cells provided apparent molecular weights for the EGF and IGF-1 receptor complexes of 165 kDa (EGF) and 290 and 135 kDa (IGF-1) (Fig. 1, lanes 4 and 7). These results were in agreement with previous findings [37, 38]. An excess of cold EGF and IGF-1 displaced the labeled EGF and IGF-1 from their receptors (Fig. 1, lanes 5 and 8). CMDB (50 μM) did not affect the binding of IGF-1 and EGF to their receptors (Fig. 1, lanes 6 and 9). Thus, these cross-linking experiments provided clear evidence that functionalized CMDB dextrans affected the binding of FGF-2 to its receptor. No such effects were observed for IGF-1 and EGF.

Increase in CMDB Fluorescence Anisotropy by FGF-2 Binding

Fluorescence anisotropy, related to the dynamic of a system, was successfully used for the measure of the apparent dissociation constants [39]. The binding of CMDB and *f*-CMDB to FGF-2 was assessed using fluorescence properties of the labeled versions of these compounds. Interactions of FGF-2 with CMDB fragments of low molecular weights (*f*-CMDB) were analyzed for their higher inhibition effect on mammary tumor cell growth compared with

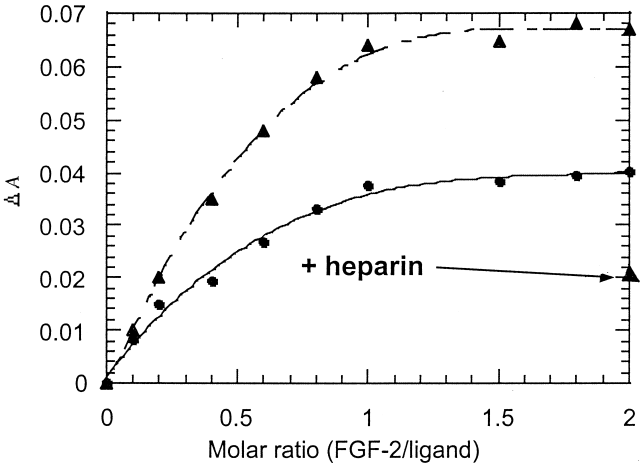

FIG. 2. FGF-2 binding to CMDB (●) and to a CMDB fragment *f*-CMDB (▲) of low molecular weight. The binding was monitored by changes in fluorescence anisotropy, a function of the molar ratio of FGF-2 to ligand. (See Materials and Methods). The CMDB concentration was fixed at 100 nM and the fluorescence monitored upon addition of FGF-2. ΔA represents the difference between the measured anisotropy at a given concentration of added FGF-2 and the initial anisotropy of CMDB. The best fit analysis yielded a calculated dissociation constant of 20 ± 10 nM. The non-linear least square procedure used for the best fit of the curve was: $y = M_0 + M_1*x + M_2*x^2 + M_3*x^3$, where $M_0 = -0.0012$, $M_1 = 0.064$, $M_2 = -0.036$ and $M_3 = 0.007$ for CMDB, and where $M_0 = -0.008$, $M_1 = 0.118$, $M_2 = -0.068$ and $M_3 = 0.012$) for *f*-CMDB. The arrow indicates the addition of a 40-fold excess of heparin.

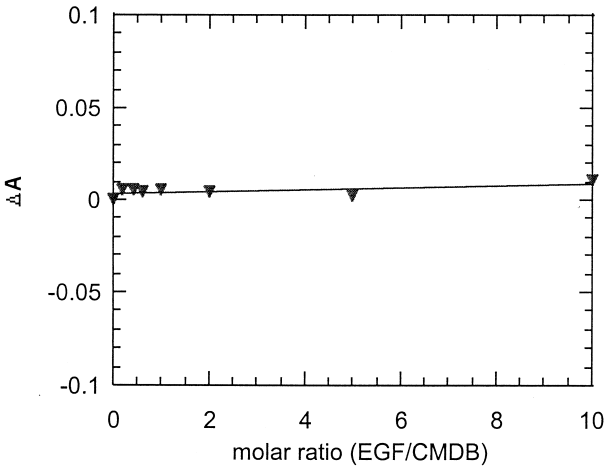

FIG. 3. Fluorescence anisotropy of labeled CMDB (100 nM) in the presence of EGF. The experiment was conducted as described for FGF-2. The molar ratio of [EGF]/[CMDB] ranges from 0 to 10.

longer fragments.* Upon excitation at 488 nm, there was a significant enhancement of fluorescence anisotropy at 517 nm. We did not observe any decrease in fluorescence quantum yield upon binding of the protein to CMDB. Figure 2 shows that the binding of FGF-2 to CMDB increased anisotropy as a consequence of the formation of a molecular complex of larger hydrodynamic volume [39]. Upon titration of CMDB with FGF-2, fluorescence anisotropy increased progressively until reaching a plateau at a molar ratio of about 1:1. Interestingly, *f*-CMDB also interacted with FGF-2 (Fig. 2). This anisotropy returned to its initial value when a molar excess (× 40) of heparin was added, while no change was observed even in the presence of a molar excess of EGF or IFG-1, reflecting the inability of these two factors to bind CMDB, as represented in Fig. 3 for EGF.

CMDB Induced Conformational Change by CD Measurement

CD measurements of FGF-2, FGF-1, IGF-1, and EGF were performed in the presence and absence of CMDB or heparin to detect the possible protein conformational changes induced by CMDB. CMDB did not contribute significantly to the spectrum. Effects are illustrated on the CD spectra reported in Fig. 4, a and b. The spectrum of FGF-2 alone contained a negative band near 205 nm and a positive band near 230 nm (Fig. 4a). This spectrum is representative of large contents of unordered and β-sheet secondary structures in FGF-2 [40]. Signal deconvolution analysis by the method of Yang [41] provides a β-sheet content as large as 60%. Addition of CMDB caused a progressive change in the CD spectrum of FGF-2, consisting mainly in a diminution of the 205 and 230 nm band intensities (Fig. 4a). In comparison, heparin exerted a more significant effect on FGF-2. Measure of the ellipticity at 230 nm gave an indication of the progressive change occurring in the conformation of FGF-2 through addition of CMDB or heparin (Fig. 4b). Saturation occurred at a molar ratio of 1:1 for both compounds. The same molar ratio was found by fluorescence analysis. The precursors of CMDB, i.e. dextran and carboxymethyl dextran (CMD), had no effect on the CD spectrum of FGF-2 (data not shown). Figure 4c shows the CD spectrum of EGF. This spectrum contains a negative band near 200 nm, a weak positive band near 235 nm, and a positive band below 190 nm. Signal deconvolution analysis provided a large β-sheet content (55%). We noted that EGF (as well as IGF-1), which is reported to be a potent stimulator of mammary cell growth [42, 43], did not present any CD variation when mixed with CMDB (Fig. 4c). The same was true for IGF-1 and FGF-1 (data not shown).

Binding Affinity and Stoichiometry of FGF-2–CMDB Complexes

The binding properties of CMDB to FGF-2, IGF-1, and EGF were further assessed by fluorescence anisotropy experiments. Binding constants can be estimated from the curve of fluorescence anisotropy variation (Fig. 2). When binding occurs, anisotropy varies with the amount of protein added to CMDB, according to a Scatchard model

* Bittoun P, Chaubet F, Jozefonvicz J and Fermandjian S, unpublished results.

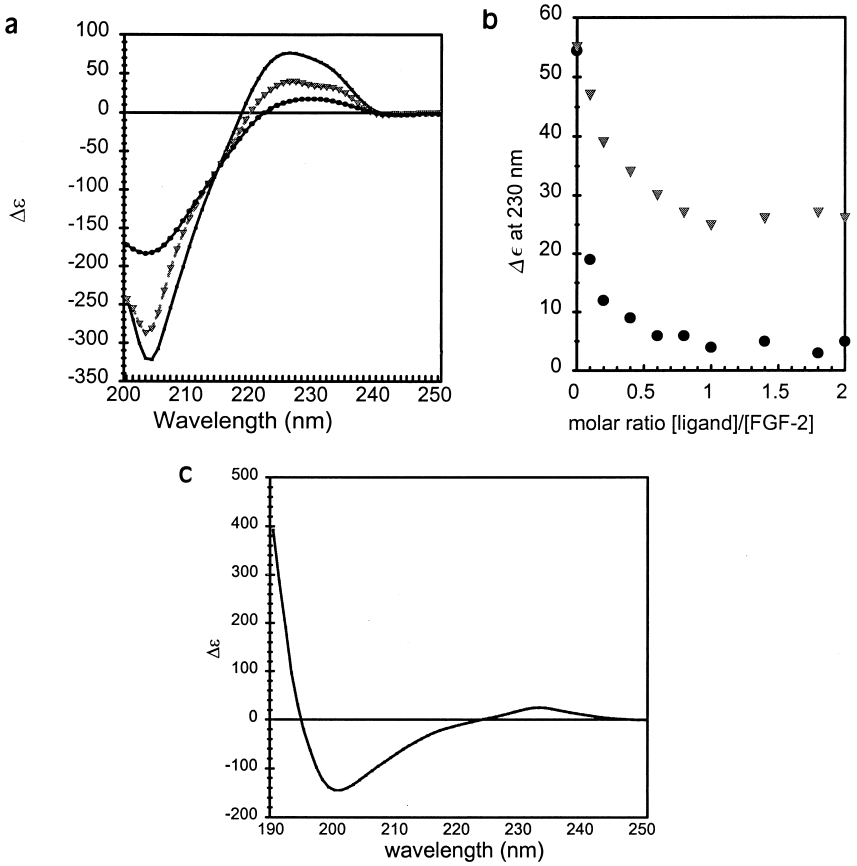

FIG. 4. CD experiments: (a) CD spectra of FGF-2 alone (—) and in the presence of CMDB (▼) and heparin (●); (b) Δε changes at 230 nm for FGF-2 as a function of addition of CMDB (▼) and heparin (●); and (c) CD spectrum of EGF alone (—) and in the presence of CMDB (▼). CD spectra of FGF-2 and EGF were measured in Tris–HCl buffer (10 mM NaCl, pH 7.4) in the peptide region as a function of CMDB to FGF-2 molar ratio. Spectra were recorded in 0.1-cm cells at a protein concentration of 5 μM. The contribution of CMDB was subtracted. Units are Δε ($cm^{-1}.M^{-1}$).

for an independent set of binding sites and assuming that one FGF-2 molecule binds to one molecule of CMDB or *f*-CMDB. At saturation, the total concentration of the labeled CMDB gives the total concentration of FGF-2.

The binding of CMDB was measured by change in fluorescence intensity according to previous studies [39, 44]. The change in polarization parallels the change in fluorescence intensity. Binding constants were estimated from labeled CMDB or *f*-CMDB using non-linear least square computer fit to the line through the anisotropy data [39]. Changes in fluorescence anisotropy are presented here. The apparent K_d for binding of CMDB to FGF-2 was found to be 20 ± 10 nM. The stoichiometry of FGF-2 complexed to the ligand was determined by titrating labeled CMDB (Mr = 80000 g/mol) or *f*-CMDB (6000 g/mol) at a fixed concentration of 100 nM, that is above the measured dissociation constant (20 ± 10 nM). Under these conditions, the titration reached a clear end point, allowing a direct measurement of the interaction stoichiometry. We found that 1 mole of FGF-2 bound molecule of CMDB. With *f*-CMDB (6,000 g/mol), no difference was observed compared to CMDB.

DISCUSSION

CMDB are functionalized dextrans exhibiting some of the biological properties of heparin. We have previously shown that these polymers are able to inhibit human mammary tumor cell growth [24, 26] and have proposed that a main CMDB effect is mediated through a direct interaction with FGF-2 [1]. Here, we have shown the capacity of CMDB to selectively displace FGF-2 from its receptor. In contrast, CMDB did not affect the binding of IGF-1 and EGF to their receptors, although these two growth factors are, like FGF-2, good enhancers of mammary cell growth [42, 43]. These results account for the differential affinities of CMDB for growth factors.

The direct interaction of CMDB with FGF-2 is supported by both anisotropy fluorescence and CD analysis. The binding of CMDB to growth factors could be detected by monitoring changes in the fluorescence anisotropy of a fluorophore attached to CMDB. Of all the growth factors tested, only FGF-2 promoted a significant increase in labeled CMDB anisotropy, indicating the formation of a molecular complex of clearly larger hydrodynamic volume. Moreover, heparin competed with CMDB for binding to FGF-2, signifying that CMDB and heparin could bind at the same site on FGF-2. This finding is supported in our experiments by a return of the anisotropy to its initial value in the presence of a molar excess of heparin. Comparatively, anisotropy remained unchanged upon the addition of IGF-1 or EGF to CMDB, indicating that no binding had occurred and confirming the result of cross-link experiments. EGF did not bind either to heparin or to glycosaminoglycans, as described in earlier studies [45, 46].

CD measurements revealed that both CMDB and hepa-

rin induced a conformational change in FGF-2. In contrast, dextran molecules failed to exert any effect on FGF-2, in agreement with previous infrared spectroscopy results [10]. Analysis of CD spectra, using the method of Yang [41], showed that FGF-2 is mainly composed of β-sheet structure (60%), as shown in earlier studies [47–49]. Addition of CMDB to FGF-2 decreased its β-sheet content, although to a lesser extent than with heparin. In contrast, both IGF-1 and EGF, despite their large fraction of β-sheet, did not reflect structural variations in the presence of CMDB or heparin. It is noteworthy that FGF-1, which shares significant sequence homology and structure with FGF-2, did not present any structural variation upon the addition of CMDB or heparin (not shown). This confirmed previous CD studies on FGF-1 mixed with heparin [45].

Taken together, cross-linking studies, fluorescence anisotropy, and CD experiments account for the specificity of CMDB towards FGF-2. Heparin interacts with particular residues of a consensus sequence commonly found in the so-called heparin-binding proteins, including lysines shown to play a crucial role in the affinity of FGF-2 towards heparin [13, 49, 50–52]. Both EGF and IGF-1, which do not bind heparin, are lacking in such a basic domain. However, the basic domain is not sufficient in itself for the binding of glycosaminoglycans to proteins [14]. As expected, the secondary and tertiary structures also interfere with the affinity of proteins for glycosaminoglycans [16]. For instance, FGF-1 and FGF-4, which are heparin-binding proteins with a basic domain [2], did not present binding properties towards CMDB in our study (data not shown).

Guimond *et al.* [46] have shown that the 2- and 6 -*O*-sulfate groups of heparin are required for an efficient mitogenic activity. The carboxyl groups of uronic acids, important for heparin-binding activity, can be replaced by any other functional group with a negative charge, including amidomethyl sulfonate [22]. Moreover, Ornitz *et al.* have found that a non-sulfated trisaccharide can bind to the higher affinity region of FGF-2 by using only carboxyl and hydroxyl group interactions with protein residues [53]. In the case of CMDB, the negatively charged carboxymethyl groups attached mainly at the C2 position of the glycosidic ring [54] could interact with the FGF-2 heparin-binding site, thereby playing the role of the heparin *O*-sulfate groups. Moreover, the carboxymethyl and benzylamide groups of CMDB molecules are involved in the inhibition of mammary tumor cell growth [25, 26]. The fact that dextrans and CMD (carboxymethyl dextran) do not bind to FGF-2 strongly supports a participation of the benzylamide group of CMDB in the interaction with FGF-2.

In contrast, no difference either in affinity (K_d: 20 ± 10 nM) or stoichiometry (1:1) was observed between CMDB (80,000 g/mol) and *f*-CMDB (6,000 g/mol) binding to FGF-2. CMDB and *f*-CMDB display the same CM and B overall composition, but do not inhibit mammary tumor cell growth with the same efficiency. In our recent experiments, *f*-CMDB with molecular weights from 5,000 to 20,000 g/mol have been found to be more potent inhibitors than the parent CMDB, although they bind FGF-2 equally well. This difference could be partly explained by the fact that *f*-CMDB are internalized into cells, whereas the larger CMDB are not.*

Finally, CMDB emerge as a class of growth factor antagonists blocking in particular FGF-2 mitogenic activity. It is thus proposed that high and low molecular weight CMDB encompass a heparin-like sequence needed for their binding to FGF-2 and their mitogenic activity. The direct interactions of CMDB with FGF-2 are responsible for conformational changes in FGF-2, altering its receptor-binding properties. *f*-CMDB can exert their inhibitory effects either at the cell perimeter or inside the cell, perhaps on the nucleus.

Indeed, *f*-CMDB are better inhibitors of mammary cell growth compared to full-size CMDB.* Such a differential growth effect occurring without a variation in binding affinity or stoichiometry could reflect a more efficient internalization of short *f*-CMDB in the cells compared to CMDB.

References

1. Bagheri-Yarmand R, Liu JF, Ledoux D, Morère JF and Crépin M, Inhibition of human breast epithelial HBL100 cell proliferation by a dextran derivative (CMDB7): Interference with the FGF-2 autocrine loop. *Biochem Biophys Res Commun* **239:** 424–428, 1997.
2. Burgess WH and Maciag T, The heparin-binding fibroblast growth factor family of proteins. *Annu Rev Biochem* **58:** 575–606, 1989.
3. Basilico C and Moscatelli D, The FGF family of growth factors and oncogenes. *Adv Cancer Res* **59:** 115–165, 1992.
4. Moscatelli D, Joseph-Silverstein J, Presta M and Rifkin DB, Multiple forms of an angiogenesis factor: Basic fibroblast growth factor. *Biochimie* **70:** 83–87, 1988.
5. Klagsbrun M and Baird A, A dual receptor system is required for FGF-2 activity. *Cell* **67:** 229–231, 1991.
6. Kiefer MC, Ishihara M, Swiedler SJ, Crawford K, Stephans JC and Barr PJ, The molecular biology of heparan sulfate fibroblast growth factor receptors. *Ann NY Acad Sci* **638:** 167–176, 1991.
7. Yayon A, Klagsbrun M, Esko JD, Leder P and Ornitz DM, Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high-affinity receptor. *Cell* **64:** 841–848, 1991.
8. Rapraeger AC, Krufka A and Olwin BB, Requirement of heparan sulfate for FGF-2-mediated fibroblast growth and myoblast differentiation. *Science* **252:** 1705–1708, 1991.
9. Busby TF, Argraves WS, Brew SA, Pechik I, Gilliland GL and Ingham KC, Heparin binding by fibronectin module III-13 involves six discontinuous basic residues brought together to form a cationic cradle. *J Biol Chem* **270:** 18558–18562, 1995.
10. Flaumenhaft R, Moscatelli D and Rifkin DB, Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor. *J Cell Biol* **111:** 1651–1659, 1990.
11. Prestrelski SJ, Fox GM and Arakawa T, Binding of heparin to

* Bittoun P, Chaubet F, Jozefonvicz J and Fermandjian S, unpublished results.

basic fibroblast growth factor induces a conformational change. *Arch Biochem Biophys* **293:** 314–319, 1992.
12. Lee MK and Lander AD, Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: Development of a sensitive electrophoretic approach. *Proc Natl Acad Sci USA* **88:** 2768–2772, 1991.
13. Jackson RL, Busch SJ and Cardin AD, Glycosaminoglycans: Molecular properties, protein interactions and role in physiological processes. *Physiol Rev* **71:** 481–539, 1991.
14. Thompson LD, Pantoliano MW and Springer BA, Energetic characterization of the basic fibroblast growth factor–heparin interaction: Identification of the heparin-binding domain. *Biochemistry* **33:** 3831–3840, 1994.
15. Klagsbrun M and Shing Y, Heparin affinity of anionic and cationic capillary endothelial cell growth factor: Analysis of hypothalamus-derived growth factors and fibroblast growth factors. *Proc Natl Acad Sci USA* **82:** 805–809, 1985.
16. Ingham KC, Brew SA, Migliorini MM and Busby TF, Binding of heparin by type III domains and peptides from the carboxy terminal hep-2 region of fibronectin. *Biochemistry* **32:** 12548–12553, 1993.
17. Tyrrell DJ, Ishihara M, Rao N, Horne A, Kiefer MC, Stauber GB, Lam LH and Stack RJ, Structure and biological activities of a heparin-derived hexasaccharide with high affinity for basic fibroblast growth factor. *J Biol Chem* **268:** 4684–4689, 1993.
18. Ishihara M, Tyrrell DJ, Staubert GB, Brown S, Cousens LS and Stack RJ, Preparation of affinity-fractionated, heparin-derived oligosaccharides and their effects on selected biological activities mediated by basic fibroblast growth factor. *J Biol Chem* **268:** 4675–4683, 1993.
19. Li LY and Seddon AP, Fluorospectrometric analysis of heparin interaction with fibroblast growth factor. *Growth Factors* **11:** 1–7, 1994.
20. Turnbull JE, Fernig DG, Ke Y, Wilkinson MC and Gallagher J, Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J Biol Chem* **267:** 10337–10341, 1992.
21. Ishihara M, Tyrrell DJ, Kiefer MC, Barr PJ and Swiedler SJ, A cell-based assay for evaluating the interaction of heparin-like molecules and fibroblast growth factor. *Anal Biochem* **202:** 310–315, 1992.
22. Maccarana M, Casu B and Lindahl U, Minimal sequence in heparin/heparan sulfate required for binding of fibroblast growth factor. *J Biol Chem* **268:** 23898–23905, 1993.
23. Ishihara M, Shaklee PM, Yang Z, Liang W, Wei Z, Stack R and Holme K, Structural features in heparin which modulate specific biological activities mediated by basic fibroblast growth factor. *Glycobiology* **4:** 451–458, 1994.
24. Mauzac M and Jozefonvicz J, Anticoagulant activity of dextran derivatives. Part I: Synthesis and characterization. *Biomaterials* **5:** 301–304, 1984.
25. Bagheri-Yarmand R, Morère JF, Letourneur D, Jozefonvicz J, Israel L and Crépin M, Inhibitory effects of dextran derivatives *in vitro* on the growth characteristics of premalignant and malignant human mammary epithelial cell lines. *Anticancer Res* **12:** 1646, 1992.
26. Bagheri-Yarmand R, Bittoun P, Champion J, Letourneur D, Jozefonvicz J, Fermandjian S and Crépin M, Carboxymethyl benzylamide dextrans inhibit breast cancer cell lines. In Vitro *Cell Dev Biol* **30a:** 822–824, 1994.
27. Letourneur D, Champion J, Slaoui F and Jozefonvicz J, *In vitro* stimulation of human endothelial cells by derivatized dextrans. In Vitro *Cell Dev Biol* **29a:** 67–73, 1993.
28. Letourneur D, Logeart D, Avramoglou T and Jozefonvicz J, Antiproliferative capacity of synthetic dextrans on smooth muscle cell growth: The model of derivatized dextrans as heparin-like polymers. *J Biomater Sci Polym Edn* **4:** 431–434, 1993.
29. Chaubet F, Champion J, Maïga R, Maurey S and Jozefonvicz J, Synthesis and structure–anticoagulant property relationships of functionalized dextrans: CMDBS. *Carbohyd polym* **28:** 145–152, 1995.
30. Tardieu M, Slaoui F, Jozefonvicz J, Courty C and Barritault D, Biological and binding studies of acidic fibroblast growth factor in the presence of substituted dextran. *J Biomater Sci Polym Edn* **1:** 63–70, 1989.
31. Tardieu M, Gamby C, Avramoglou T, Jozefonvicz J and Barritault D, Derivatized dextrans mimic heparin as stabilizers, potentiators, and protectors of acidic or basic FGF. *J Cell Physiol* **150:** 194–203, 1992.
32. Maïga-Revel O, Chaubet F and Jozefonvicz J, New investigation on heparin-like derivatized dextrans: CMDBS, Synergistic role of benzylamide and sulfate subsituents in anticoagulant activity. *Carbohydr Polym* **32:** 89–93, 1997.
33. Stearns NA, Prigent-Richard S, Letourneur D and Castellot JJ Jr, Synthesis and characterization of highly sensitive heparin probes for detection of heparin-binding proteins. *Anal Biochem* **247:** 348–356, 1997.
34. Nardella A, Chaubet F, Boisson-Vidal C, Blondin C, Durand P and Jozefonvicz J, Anticoagulant low molecular weight fucans produced by radical process and ion exchange chromatography of high molecular weight fucans extracted from brown seaweed *Ascophyllum nodosum*. *Carbohydr Res* **289:** 201–208, 1996.
35. Lee YC, Fluorescence spectrometry in studies of carbohydrate–protein interactions. *J Biochem* **121:** 818–825, 1997.
36. Moenner M, Zaki-Gannoun L, Badet J and Barritault D, Internalization and limited processing of basic fibroblast growth factor on chinese hamster lung fibroblasts. *Growth Factors* **1:** 115–123, 1989.
37. Fitzpatrick SL, LaChance MP and Schultz GS, Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. *Cancer Res* **44:** 3442–3447, 1984.
38. Osborne CK, Coronado EB, Kitten LJ, Arteaga CI, Fuqua SA, Ramasharma K, Marshall M and Li CH, Insulin-like growth factor II (IGFII): A potent autocrine/paracrine growth factor for human breast cancer acting via the IGF receptor. *Mol Endocrinol* **3:** 1701–1709, 1989.
39. Zhuang P, Chen AL and Peterson CB, Native and multimeric vitronectin exhibit similar activity for heparin. *J Biol Chem* **272:** 6858–6867, 1997.
40. Wu C-SC, Thompson SA and Yang JT, Basic fibroblast growth factor is a β-rich region. *J Protein Chem* **10:** 427–436, 1991.
41. Yang JT, Wu CSC and Martinez H, Calculation of protein conformation from circular dichroism. *Methods Enzymol* **110:** 208–230, 1986.
42. Sallé V, Raux H, Souttou B, Israël L and Crépin M, Primary cultures of human benign mastopathies and mammary carcinoma growth factor requirements. *Anticancer Res* **11:** 895–900, 1991.
43. Huff KK, Kaufman D, Gabby KH, Spencer EM, Lippman ME and Dickson RB, Secretion of an insulinic-like growth factor-1-related protein by human breast cancer cells. *Cancer Res* **46:** 4613–4619, 1986.
44. Taira K and Benkovic S, Evaluation of the importance of hydrophobic interactions in drug binding to dihydrofolate reductase. *J Med Chem* **31:** 129–137, 1988.
45. Copeland RA, Hanlee J, Halfpenny AJ, Williams RW, Thompson KC, Herber WK, Thomas KA, Brunner MW, Ryan JA, Marquis-Omer D, Sanyal G, Sitrin RD, Yamazaki S and Middaugh CR, The structure of human acidic fibroblast

growth factor and its interaction with heparin. *Arch Biochem Biophys* **289:** 53–61, 1991.
46. Guimond S, Maccarana M, Olwin BB, Lindhal U and Rapraeger AC, Activating and inhibitoring heparin sequences for FGF-2 (basic FGF). Distinct requirements for FGF-1, FGF-2, and FGF-4. *J Biol Chem* **268:** 23906–23914, 1993.
47. Fox GM, Schiffer SG, Rohde MF, Tsai LB, Banks AR and Arakawa T, Production, biological activity, and structure of recombinant basic fibroblast growth factor and an analog with cysteine replaced by serine. *J Biol Chem* **263:** 18452–18458, 1988.
48. Arakawa T, Hsu YR, Schiffer SG, Tsai LB, Curless C and Fox GM, Characterization of a cysteine-free analog of recombinant human basic fibroblast growth factor. *Biochem Biophys Res Commun* **161:** 335–341, 1989.
49. Zhu X, Komiya H, Chirino A, Faham S, Fox GM, Arakawa T, Hsu BT and Rees DC, Three-dimensional structures of acidic and basic fibroblast growth factors. *Science* **251:** 90–93, 1991.
50. Eriksson EA, Cousens LS, Weaver LH and Matthews BW, Three-dimensional structure of human basic fibroblast growth factor. *Proc Natl Acad Sci USA* **88:** 3441–3445, 1991.
51. Zhang JD, Cousens LS, Barr PJ and Sprang SR, Three-dimentional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1β. *Proc Natl Acad Sci USA* **88:** 3446–3450, 1991.
52. Faham S, Hileman RE, Fromm JR, Linhardt RJ and Rees DC, Heparin structure and interactions with basic fibroblast growth factor. *Science* **271:** 1116–1120, 1996.
53. Ornitz DM, Herr AB, Nilsson M, Westman J, Svahn CM and Walksman G, FGF binding and FGF receptor activation by synthetic heparan-derived di- and trisaccharides. *Science* **268:** 432–443, 1995.
54. Krentsel L, Chaubet F, Rebrov A, Champion J, Ermakov I, Bittoun P, Fermandjian S, Litmanovitch A, Platé N and Jozefonvicz J, Anticoagulant activity of functionalized dextrans. Structure analyses of carboxymethylated dextran and first Monte-Carlo simulations. *Carbohyd Polym* **33:** 63–71, 1997.