Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 290-296

DISTRIBUTION OF BASIC FIBROBLAST GROWTH FACTOR BINDING SITES IN VARIOUS TISSUE MEMBRANE PREPARATIONS FROM ADULT GUINEA PIG

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Received December 21, 1988

Summary : In order to localize a rich source of basic FGF receptor, we examined the distribution of basic FGF binding sites in brain, stomach, lung, spleen, kidney, liver and intestine membrane preparations from adult guinea pig. Comparative binding studies using iodinated basic FGF showed that a specific binding was detected in all the membrane preparations tested. Scatchard plots from iodinated basic FGF competition experiment with native basic FGF in various membrane preparations, suggested the presence of one class of binding sites in some tissues such as liver, kidney, spleen, lung, stomach, and intestine with an apparent dissociation constant ($_{app}$KD) value ranging from 4 to 7.5 nM and the existence of a second class of higher affinity sites in brain membranes with $_{app}$KD value of 15 pM. Characterization of these basic FGF high affinity interaction sites was performed using a cross-linking reagent. These results show for the first time that specific interaction sites for basic FGF are widely distributed, suggesting that this growth factor might play a role in the physiological functions of a number of adult organs.

Introduction : Basic FGF , a 17 kDa heparin-binding growth factor, analogous to eye-derived growth factor I (1), induce *in vitro* the proliferation of a wide variety of cells (2, 3). One of the first steps of induction of the mitogenic signal is a specific interaction with high affinity cell membrane receptors that we and other groups have identified on various cell types including bovine epithelial lens cells (4), mouse muscle myoblast, 3T3 cells (5), baby hamster kidney cells (6) and endothelial cells (7). More recently, several reports have described the existence of a second cell membrane-associated binding site (8, 9, 10) which might carry cell-associated heparin like molecules (8, 9).

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Abbreviations : Basic FGF : basic fibroblast growth factor. DSS : dissuccinimidyl suberate. SDS : sodium dodecyl sulfate. HEPES : 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid. BSA : bovine serum albumin.

0006-291X/89 $1.50

The physiological function of this class of low affinity sites is still unknown. However, basic FGF has a strong affinity for heparin or heparin-like molecules (2, 8, 11, 12), a property that has been used for its purification (1, 2, 3). Since heparin or heparin like structures have been shown to stabilize the biological activity of basic FGF *in vitro* (13, 14), it was tempting to suggest that these low affinity membrane associated binding sites might represent *in vivo* a mean of storage and/or protection for basic FGF (15), from which this factor will be readily available. The ubiquitous tissue distribution of basic FGF prompted us to study in this work the distribution of low andhigh affinity binding sites of basic FGF in tissue membrane preparations from various organs.

We present here the results obtained with brain, stomach, lung, spleen, kidney, liver and intestine membrane preparations from adult guinea pig using the assay that we have recently described for bovine brain membranes (16).

MATERIALS AND METHODS

- Materials : [^{125}I]Na was obtained from Oris France, disuccinimidyl suberate from Pierce Chemical Co. High molecular weight standards and all the reagents used for SDS-Polyacrylamide Gel Electrophoresis were obtained from Bio-Rad. Basic FGF was purified from bovine brain using heparin-Sepharose chromatography as previously described (1).

- Iodination of basic FGF : Basic FGF was iodinated using the chloramine-T method (17) with the following modifications. Briefly, 3 ug of basic FGF in 0.1 M phosphate buffer (pH 7.4) containing 1 % polyethylene glycol-1000 were incubated at room temperature for 2 min. with 1 mCi of [^{125}I]Na and 20 µM of Chloramine T. After addition of 100 mM N-acetyltyrosine, free iodine was eliminated by heparin-Sepharose chromatography. The biological activity was controlled as previously described (18) and [^{125}I]basic FGF was stored at 4°C for two weeks without significant loss of the biological activity. The specific activity obtained was ranging from 1.7 x 10^6 to 3.4 x 10^6 cpm/pmol.

- Tissue membrane preparations : Tissue membrane preparations were prepared according to a procedure previously described (16). Briefly, fresh brain, intestine, spleen, liver, stomach, kidney and lung were carefully dissected from anaesthetized adult female guinea pig (250g), weighed, cut into small pieces and immediately homogeneized in 2 volumes of 20 mM Hepes (pH 7.4) containing 0.3 M sucrose, 5 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 5 Kallikrein Inhibitor Units (KIU) per ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (Buffer A) using an Ultra Turax homogenizer. The suspension was centrifuged (1,000 g, 4°C for 15 min.) and the resulting supernatant was then pelleted for 15 min. at 40,000 g. This pellet was resuspended by 2 volumes of buffer A containing 3 M $MgCl_2$, incubated 30 min. at 4°C and washed twice in buffer B (buffer A containing 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1 M NaCl without EDTA) by repeated centrifugations. The final pellet was resuspended in buffer B, adjusted to a protein concentration of 1 mg/ml and stored at -80 °C until used.

- Binding assay : Binding assay was performed using the procedure previously described (16). Briefly, 15 µg of total proteins for lung and 40 µg for the other tissues were incubated for 60 min. at 4° C with 200 pg (23 pM) of iodinated basic FGF in the presence or absence of unlabeled basic FGF (300 ng) in a final volume of 500 µl of buffer B containing 0.5% BSA. After incubation, bound and free basic FGF were separated by centrifugation (5,600 g, 5 min. at 4° C). The resulting pellet containing bound basic FGF was placed in a gamma counter and the radioactivity measured.

Non specific binding was estimated in the presence of an excess of unlabeled basic FGF and represented about 12-23% of the total added radioactivity according to the tissues used. Scatchard analysis of the data obtained from the isotopic dilution of [^{125}I]basic FGF by various concentrations of basic FGF ranging from 10^{-11} to 4 x 10^{-8} M were obtained using the LIGAND fitting program (19).

- Affinity Cross-linking Experiment : 100 µg of the equivalent of proteins from each of the tissue membrane preparations were incubated in buffer B for 1 hour at 4°C with 10^6 cpm of basic FGF in absence or in presence of a 100-fold molar excess of unlabeled basic FGF. The cross-linking reaction was then initiated by adding 0.1 mM DSS. After 15 min. of incubation at room temperature, the reaction was quenched by the addition of 10 mM methylamine. Cross-linked membranes were washed with buffer B by repeated centrifugations (5,600 g, 5 min. at 4°C) and solubilized by incubation for 15 min. in a buffer containing 2% SDS, 10% glycerol, 70 mM Tris (pH 6.8). Unsolubilized materials were pelleted by centrifugation ; analysis of the resulting supernatant was performed by SDS-Polyacrylamide gel electrophoresis in a 5-7% gradient according to the procedure described by Laemmli (20) and autoradiography at -80°C using Kodak X-Omat R film.

- Protein Determination : The protein content was determinated using the BCA Protein Assay Reagent (21) with bovine serum albumin as a standard.

RESULTS

Presence of specific binding of iodinated basic FGF in various tissue membrane preparations. In order to determined the existence of specific binding of [^{125}I]basic FGF in membrane preparations from various tissues of adult guinea pig such as kidney, brain, lung, spleen, intestine, liver and stomach, 200 pg (23 pM) of iodinated basic FGF in presence or not of an excess of unlabeled basic FGF (300 ng) were incubated 60 min. at 4°C with 15 µg of lung membrane preparations and 40 µg of other membrane preparations considered in this study. This period of incubation and the amount of tissue membranes used corresponded to the maximum specific binding observed (result not shown). Distribution of [^{125}I]basic FGF binding in various tissue membrane preparations is shown on Fig. 1. Each of the preparation exhibited [^{125}I]basic FGF binding that was displaced by an excess of native basic FGF. Brain membrane preparations showed the highest specific binding (Student test with $p < 0.03$), representing 35 ± 3 % of total added radioactivity.

Analysis of the interaction sites of basic FGF to tissue membrane preparations. Analysis of the interaction sites of basic FGF to tissue membrane preparations were performed using Scatchard transform of iodinated basic FGF isotopic dilution by native basic FGF as previously described for bovine brain membranes. Using the LIGAND fitting program, Scatchard transform from each isotopic dilution curve (not shown) indicated that only data obtained from brain membranes were non linear, suggesting the presence of at least two families of interaction sites in this membrane preparation (Fig.2). Table 1 gives a comparative summary of equilibrium parameters of basic FGF. Kidney, liver, lung, intestine, spleen and stomach membrane preparations exhibited a single class of affinity with apparent dissociation constant ($_{app}$KD) ranging from 4 to 7.5 nM (n=4) and a binding capacity ranging from 27 to 56 pmoles per mg

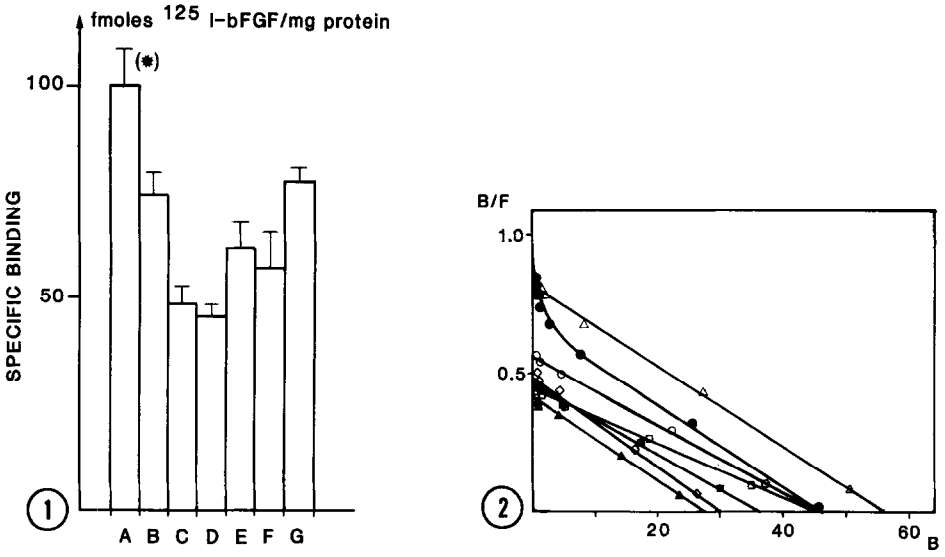

Figure 1 - Distribution of [^{125}I]basic FGF binding to membranes prepared from various organs of guinea pig. Membranes were prepared from brain (A), intestine (B), kidney (C), liver (D), lung (E), spleen (F) and stomach (G) of adult guinea pig. Membrane preparations were incubated for 60 min. at 4°C with 200 pg (23 pM) of iodinated basic FGF in presence (non specific binding) or in absence (total binding) of 300 ng of unlabeled basic FGF. Bound basic FGF was determinated as described in "Materials and Methods". Data shown represented the mean values ± SD of the specific binding obtained, expressed in fmoles of [^{125}I]basic FGF bound per mg of membrane protein of four separate experiments, each in duplicate.
(*):Value significantly different (Student test with $p < 0.03$) from values obtained for other membrane preparations.

Figure 2 - Scatchard analysis of the data derived from the binding competition studies. Membrane preparations obtained from brain (●), intestine (◇), kidney (■), liver (□), lung (○), spleen (▲) and stomach (△) were incubated 60 min. at 4°C with iodinated basic FGF in presence or not of increasing concentrations of native basic FGF ranging from 10^{-11} to 4×10^{-8} M. Bound radioactivity was measured as described in "Materials and Methods". Scatchard analysis of the data obtained from the binding competition studies were performed using the curve-fitting LIGAND program. B/F represents the bound/free ratio of the ligand and B represents specific bound ligand in pmoles/mg of membrane protein. Data shown represent computer estimation of four separate experiments resulting from three different tissue membrane preparations.

Table 1 - Apparent dissociation constants for basic FGF interactions with high and low affinity sites on various membrane preparations of adult guinea pig

	HIGH AFFINITY		LOW AFFINITY	
TISSUES	$_{app}$KD (pM)	BINDING SITES (fmoles/mg)	$_{app}$KD (nM)	BINDING SITES (pmoles/mg)
BRAIN	15 ± 7[a]	40 ± 10[b]	4.0 ± 0.4[a]	42 ± 3[b]
INTESTINE	ND	ND	4.6 ± 0.6	28 ± 4
KIDNEY	ND	ND	5.7 ± 0.7	37 ± 3
LIVER	ND	ND	7.5 ± 0.7	47 ± 3
LUNG	ND	ND	5.3 ± 0.9	47 ± 4
SPLEEN	ND	ND	4.6 ± 0.6	27 ± 4
STOMACH	ND	ND	4.3 ± 0.5	56 ± 6

ND : Not detected
Calculated with the LIGAND fitting program, data shown are obtained from results presented on Fig. 2 and represent the mean values ± SD of four independent experiments.
The preciseness of the calculation is at 0.1 pM[a] and 1 fmole/mg of protein[b].

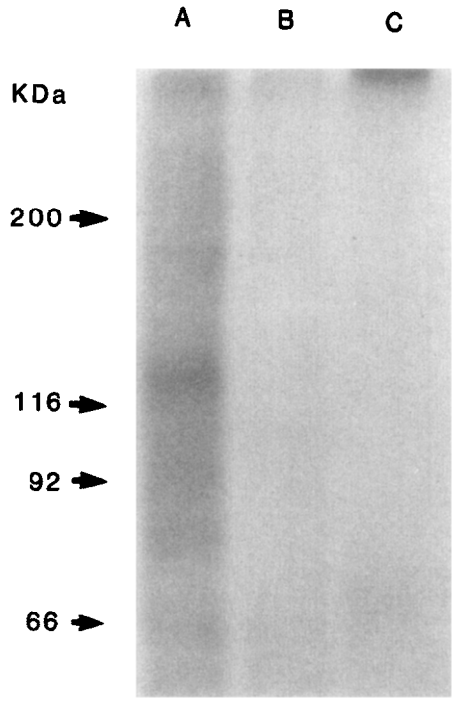

Figure 3 - Cross-linking experiments of [^{125}I]basic FGF-receptor complexes from guinea pig brain membrane preparation. 100 µg of membrane preparations were incubated for 60 min. at 4°C with 10^6 cpm of basic FGF in the presence (lane B) or not (lane A) of a 100-fold excess of native basic FGF. 0.1 mM of DSS was then added for 15 min. at room temperature and quenched with 10 mM methylamine. The cross-linked membranes were analyzed by SDS-Polyacrylamide gel electrophoresis and autoradiography. The same experiment described for lane A was run in absence of brain membrane preparations (lane C). The proteins used as molecular weight standards were myosin (200,000), β galactosidase (116,000), phosphorylase b (92,000) and bovine serum albumin (66,000).

of membrane protein. In brain membranes, additional binding sites with $_{app}$KD of 15 pM and a binding capacity of 40 fmoles per mg of membrane protein were observed.

Cross linking experiments. The apparent molecular weight of the complex formed by [^{125}I]basic FGF bound to its specific binding site was estimated by chemical cross-linking experiment for each tissue membrane preparation. The cross-linked products obtained were analysed by SDS-Polyacrylamide gel electrophoresis and autoradiography as described in "Materials and Methods". Among all membrane preparations tested, the analysis of obtained autoradiograms yielded two distinct bands with apparent molecular weights of 170 kDa and 135 kDa only in the case of brain membrane preparations as shown on Fig 3, lane A. Cross linking carried out in the presence of a 100-fold excess of unlabeled basic FGF completely prevented labeling of these two components (Fig 3, lane B).

DISCUSSION

This study reports for the first time that basic FGF interaction sites are widely distributed in guinea pig tissues. Using the same assay that we have previously described (16), Scatchard analysis of the isotopic dilution of iodinated basic FGF bound to membrane preparations from kidney, liver, lung , intestine, spleen and stomach of adult guinea pig by native basic FGF revealed that data are best fitted by a straight line. From this analysis, we can propose that basic FGF is bound to a single set of site. The value of the apparent dissociation constant observed ($_{app}Kd = 4 - 7.5$ nM) suggests that these classes of binding sites might be related to the low affinity binding sites described on various cell types in tissue culture (8, 9, 10) or on bovine brain membranes as it has also recently been reported (16). Other works indicate that these low affinity binding sites have been partially destroyed by heparinase or heparitinase treatment and therefore could represent cell – associated heparin – like molecules (8, 9). Basic FGF binds strongly to heparin (2, 3). Heparin and other related molecules such as proteoheparan sulfate when associated with FGF, act as protector against heat or acid inactivation (13, 14). Recently, basic FGF – binding heparan sulfate molecules produced *in vitro* by bovine capillary endothelial cells has been recently characterized (11). These molecules related to heparan sulfate proteoglycan protect biological activity of basic FGF from proteolytic degradation induced *in vitro* by plasmin or trypsin (11).

The physiological meaning of the existence of these interactions with heparin and heparin like molecules remains to be elucidated. Since basic FGF has been isolated from a wide variety of tissues, the presence of these low affinity heparin – like binding sites on all tissue membranes tested suggests a possible physiological role in the storage and protection of basic FGF. Hence, stored and protected basic FGF could be available, ready to be liberated by still unknown mechanisms and could therefore acceed to the functional high affinity binding sites. We have recently described that heparin – binding proteins modulated *in vitro* the mitogenic effect and the binding of basic FGF (22). The presence of these cell – associated heparin – like molecules could therefore be implicated in a possible mechanism of regulation of the biological activity of basic FGF *in vivo* such as neovascularization or wound healing. Interestingly, the presence of basic FGF high affinity sites was only observed in brain membrane preparations. Apparent affinity constants were closely related to the values measured from bovine brain membranes experiments. Characterization of the interaction site by chemical cross – linking reagent revealed that basic FGF interaction involved a 150 kDa and 115 kDa polypeptides as also described for various cell types in tissue culture (4, 6) or bovine brain membranes (16). An additional 85 kDa polypeptide was also detected and could represent a degradation product of the higher molecular weight form occuring after or before the cross – linking reaction.

The physiological meaning of the presence of this high affinity set of sites detected only in the brain is unclear. Basic FGF have been found in relatively high concentration in this tissue. The localization of high affinity basic FGF interaction sites detected only in the brain could suggest that basic FGF may act in the normal maintenance or function of the neuronal cells including glial and nervous cells present in this tissue.

ACKNOWLEDGMENTS

We thank C. Gamby for technical assistance and Dr P.J. Munson for giving the Ligand program. This work was supported by "Le ministère de l'Education Nationale, le C.N.A.M.T.S. (contrat réf. 86 2-5-4-E), l'I.N.S.E.R.M. (contrat externe réf. 87 2007), l'Association de la Recherche sur le Cancer, la Ligue National du Cancer, la Fondation de la recherche Médicale et Naturalia et Biologia". J. Courty is "chargé de recherche" from the "Centre National de la Recherche Scientifique".

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